EH 459274014US

PCT/GB99/0141

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

IRVINE, Jonquil, Claire
J. A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

A. KEMP & Co
RECD 12 MAY 2000
Action by.....

Date of mailing (day/month/year) 28 April 2000 (28.04.00)	
Applicant's or agent's file reference N.74383A JCI	**IMPORTANT NOTIFICATION**
International application No. PCT/GB99/01413	International filing date (day/month/year) 06 May 1999 (06.05.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

ISIS INNOVATION LIMITED
2 South Parks Road
Oxford OX1 3UB
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

ISIS INNOVATION LIMITED
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United Kingdom

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State of Residence: GB
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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Jean-Marie McAdams
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 17 December 1999 (17.12.99)	
International application No. PCT/GB99/01413	**Applicant's or agent's file reference** N.74383A JCI
International filing date (day/month/year) 06 May 1999 (06.05.99)	**Priority date** (day/month/year) 06 May 1998 (06.05.98)
Applicant BROWNLEE, George, Gow et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

22 November 1999 (22.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

16

PATENT COOPERATION TREATY

REC'D 23 AUG 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
N.74383A JCI		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/01413	06/05/1999	06/05/1998
International Patent Classification (IPC) or national classification and IPC C12N15/44		
Applicant ISIS INNOVATION LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/11/1999	18.05.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Steffen, P Telephone No. +49 89 2399 7307

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/01413

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-4,6-40	as originally filed			
5	as received on	19/05/2000	with letter of	17/05/2000

Claims, No.:

7-27	as originally filed			
1-6	as received on	19/05/2000	with letter of	17/05/2000

Drawings, sheets:

1/9-9/9	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6,7,10,11,19-21,23-25,27
	No:	Claims	1-5,8,9,12-18,22,26
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	26,27 (see separate sheet)

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I
Basis of the report

For the reasons as set out below, the amendments, in claims 1 and in the description on page 5, filed on 19.05.2000, have no basis in the description as originally filed and therefore introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

First is the replacement of the term "thereof" by "of said protein" is not supported by the description as originally filed. This is because no basis can be found in the application as originally filed for the functional modifications of an influenza viral protein appearing in new claim 1 and in the amended description on page 5 (please refer also to point VIII of the present report).

Second is the introduction of the term "is a non-chimeric duplex region, but" not supported by the description as originally filed and therefore not allowable under the terms of article 34(2)(b) PCT. The only reference which is made in the description to the term "chimeric" is with relation to the description of the prior art (e.g. D1, see description , page 1, last paragraph). Moreover is the document D1 not only accidentally anticipating the novelty of original claim 1, but is considered as being relevant prior art to both the questions of novelty and inventive step (see point V. of the present communication). Therefore the term "is a non-chimeric duplex region, but" is intended to disclaim the content of prior art D1 from the scope of claim 1. This in itself is not allowable. Furthermore, does a basis for this disclaimer not exist in the description as originally filed, since on page 5, lines 16-18 only "**native** influenza virus vRNA duplex region derived from..." are excluded from the "(mutated) duplex region" as referred to in claim 1. However the mutated duplex regions of D1 (D1, page 3212) cannot be considered as "native", since they are "mutant" (D1, page 3212, "We have now constructed to new mutant influenza A viruses, NA/X and NA/Y,... and Fig. 1).

In conclusion, due to the unallowed amendments filed, the present report is established on the application as originally filed.

Re Item IV

Lack of unity of invention

The present application refers to life attenuated influenza viruses, carrying different mutations in the 5'-3' terminal non-coding sequences of a viral genomic RNA segment, which allow for diminished expression of an influenza viral protein coding sequence and which cause an attenuated phenotype in mice. As will be detailed in point V of the present communication, the general common concept of invention e.g. mutations in the 5'-3' terminal non-coding sequences which cause diminished expression of an influenza viral protein coding sequence and an attenuated phenotype in mice, is not novel and inventive under the provisions of articles 33(2) and 33(3) PCT. Consequently, the present application lacks unity of invention under rule 68.1 PCT, because the different mutations in the 5'-3' terminal non-coding sequences are no longer linked to a common concept of invention by means of a special technical feature. The following inventions are found:

1. claims 1-5 and claims 8-27, all partly and claim 6 complete: with reference to attenuated influenza virus by introducing specifically the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus in the 5'-3' terminal non-coding sequences to diminish expression of an influenza viral protein coding sequence and attenuating the phenotype

2. claims 1-5 and claims 8-27, all partly and claim 7 complete: with reference to attenuated influenza virus by introducing specifically the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus as well as the U-G mutation at position 10 of the 3' terminus and the A-C mutation at position 11' at the 5' terminus in the 5'-3' terminal non-coding sequences to diminish expression of an influenza viral protein coding sequence and attenuating the phenotype.

Under the provisions of rule 68.1 PCT, examination is carried out on all parts of the application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: BERGMANN M AND MUSTER T: 'The relative amount of influenza A virus segment present in the viral particle is not affected by a reduction in replication of that segment' JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 12, December 1995 (1995-12), pages 3211-3215.

D2: EP-A-0 704 533 (HOBOM G ET AL.; BAYER AG) 3 April 1996 (1996-04-03)

The subject-matter of claims 6, 7, 10, 11, 19-21, 23-25 and 27 is not disclosed by the prior art on file and therefore meets the requirements of article 33(2) PCT.

Claims 1-5, 8, 9, 12-18, 22 and 26 lack novelty under article 33(2) PCT for the following reasons.

Novelty of claims 1-5 is anticipated by D1 (NA/Y mutant influenza A virus; abstract and page 3212, left column including figure 1). The parameters as defined in claim 4 (3-4 log reduction in plaque titre on MDCK cells, compared to wild type virus) are not specifically disclosed as such in D1, but at present it cannot be excluded that the mutant virus of D1 meets also with this requirement, especially as it meets with the requirements of claim 3 (one log reduction in plaque titre on MDBK cells, compared to wild type virus, D1 page 3214, left column, two last paragraphs). The virus of claims 8 and 9 and similarly the subject-matter of claims 12-16 and 22 is disclosed in D1 (NA/Y mutant influenza A virus; abstract and page 3212, left column including figure 1). The *ex-vivo* cell of claim 17 is also disclosed in D1 (page 3213, left column). Novelty of the vaccine and method of stimulating an immune response of claims 18 and 26 is anticipated in D1 (page 3214, left column, last paragraph and right column first and last paragraph, and table 1). It has to be noted here, that albeit in D1 an immune reaction was not monitored in mice after injection of the NA/Y mutant influenza A virus, it is evident that an immune response was triggered in those mice after injecting the attenuated virus.

In consequence, claims 1-5, 8, 9, 12-18, 22 and 26 lack novelty under article 33(2) PCT and also lack inventive activity under article 33(3) PCT.

More generally, claims 1-27 lack inventive activity under article 33(3) for the following

reasons.

The present application refers to life attenuated influenza viruses, carrying different mutations in the 5'-3' terminal non-coding sequences of a viral genomic RNA segment, which allow for diminished expression of an influenza viral protein coding sequence and which cause an attenuated phenotype in mice. Furthermore several modifications of these mutated viruses as well as uses and applications thereof are claimed.

The prior art D1 discloses such attenuated mutant influenza viruses and suggests their use as vaccines. D1 anticipates novelty of claims 1-5, 8, 9, 12-18, 22 and 26 and thus leaves it impossible to acknowledge inventive activity for these claims. Mutant influenza viruses comprising heterologous coding sequences and use thereof for pharmaceutical, vaccine or antigenic delivery purpose e.g. claims 10, 11, 19-21 and 27 are/is suggested in D2 (page 2, lines 40-50). Hence inventive activity can be acknowledged for these claims only in case they are based on novel and inventive mutant attenuated influenza viruses. The use of mutated attenuated influenza A virus as helper viruses for rescue purposes e.g. claims 23-25 is self-evident for the skilled person, once the properties of the attenuated phenotype are known. Likewise, inventive step for theses claims is only acknowledgeable, once they are based on claims which are novel and inventive.

The specific mutations which are disclosed in claims 6 and 7 (e.g. C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus and the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus combined with the U-G mutation at position 10 of the 3' terminus and the A-C mutation at position 11' at the 5' terminus, in the 5'-3' terminal non-coding sequences) represent in light of the prior art D1, alternative solutions to the provision of a life attenuated influenza virus. These specific mutations yield attenuated influenza viruses which do not show any other technical effects, with resect to viability and attenuation and likely also vaccination capacities than the NA/Y mutant influenza A virus of D1. In the absence of additional, unexpected technical effects, however, inventive activity cannot be acknowledged for claims 6 and 7, with respect to the specific mutations as referred to above.

In consequence, claims 1-27 lack inventive activity under article 33(3) PCT.

Claims 26 and 27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item VIII

Certain observations on the international application

The following objections under articles 5 and 6 PCT are raised.

Claim 1 is unclear in its wording with reference to "a functional modification thereof". It cannot be appreciated from the sentence construction if this modification refers either to "a mutated duplex region" or to "an influenza viral protein". Furthermore claim 1 is missing essential technical features to clearly define the subject-matter of said claim which leads to unclarity and which is contrary to the requirements of article 6 PCT in connection with rule 6.3(a) PCT. This is because of the term "at least one base pair substitution **such that** expression of said protein coding sequence...is reduced". Here the skilled person cannot contemplate which mutations have to be introduced into the 5'-3' duplex region so to reduce expression of said protein coding sequence and for attenuation of the virus. Clearly with the information given in claim 1, the skilled person is unable to carry out the invention, because not all mutations in the 5'-3' duplex region effectively lead to reduction in expression of the protein coding sequence and to attenuation of the virus as is outlined in the description for the D1 and D3 mutations/base pair substitutions (see example 4, page 22, lines 20-22 and more specifically example 5, page 23, lines 16-17 and example 13, pages 30-31, page 31, lines 8-10). Hence claim 1 is also prone to an objection under article 5 PCT.

The terms "functional modification thereof" and "functionally equivalent substitutions" in claims 1, 6, 7, 8 and 24 are unclear with respect to the nature of the modification/ substitution to be introduced and thus do not allow to suitably delimit the scope of these claims.

Claim 2 lacks clarity with respect to "a reduction in plaque titre". Since the amount of reduction is not specified, this term is prone to subjective interpretation, thus rendering the

scope of claim 2 unclear.

CLAIMS

1. An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or a functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of said protein-coding sequence in cells infected by said virus is reduced to give an attenuated phenotype.

2. A virus as claimed in claim 1which exhibits a reduction in plaque titre compared to the parent wild-type virus on cells of one or more type selected from Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK) cells and Vero cells.

3. A virus as claimed in claim 2 which exhibits at least about one log reduction in plaque titre compared to the parent wild type virus on MDBK cells.

4. A virus as claimed in claim 2 or claim 3 which exhibits at least about 3 to 4 log reduction in plaque titre compared to the parent wild type virus on MDCK cells and Vero cells.

5. A virus as claimed in any one of claims 1 to 4 wherein said genomic nucleic acid segment is a mutated native influenza virus genomic RNA segment.

6. A virus as claimed in any one of claims 1 to 5 which is an attenuated influenza virus of type A, wherein said nucleic acid segment is a mutated influenza A virus genomic RNA segment having the mutation C to A at position 11 from the 3'-terminus of the native parent segment and the mutation G to U at position 12' from the 5'-terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an attenuating base-pair substitution in the non-coding duplex region.

has also been shown that influenza A virus with the same base pair substitution is attenuated *in vivo* and can give rise to protective immunity against wild-type influenza A virus. Evidence suggests that such attenuation arises from reduced polyadenylation of the NA-specific mRNA. Base-pair substitution in the duplex region of a vRNA segment is thus proposed as a new general strategy for achieving attenuation of influenza viruses. Such base-pair substitution can be selected by application of known rescue systems for incorporating genetically-engineered influenza vRNA segments into viable influenza viruses as further discussed below.

In one aspect, the present invention thus provides an attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of the said protein-coding sequence in cells infected by the said virus is reduced to give an attenuated phenotype.

Mutated duplex region of an influenza virus RNA genomic segment will be understood to exclude any native influenza virus vRNA duplex region derived from a vRNA of a wild-type influenza virus of a different type.

The term "cells" in this context may encompass human and/or animals cells *in vivo* normally infected by influenza viruses. For the purpose of selection of attenuated viruses of the invention, the same term will be understood to refer to cells of a single cell type or more than one type, e.g. cultured human or non-human animal cells of one or more than one type. They may be *in vivo* cells, e.g. cells of an animal model. Cultured cells which may prove useful in the selection of attenuated viruses of the invention *in vitro* include one or more of MDBK cells, Madin-Darby canine kidney (MDCK) cells and Vero (African green monkey kidney) cells.

While an attenuated virus of the invention may have a single base-pair substitution in the duplex non-coding region of a genomic segment, it will be appreciated that such a virus may have more than one such substitution, either on the same genomic segment or different genomic segments, e.g. 2 base pair substitutions in the same genomic segment duplex region. The duplex base-pair substitution(s)

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EH459274014US

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* N.74383A JCI

Box No. I TITLE OF INVENTION

MODIFIED VIRUSES

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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☐ This person is also inventor.

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State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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United Kingdom

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
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- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: SK

State *(that is, country)* of residence: GB

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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United States of America

This person is:
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- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania
- ☒ AM Armenia
- ☒ AT Austria
- ☒ AU Australia
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina
- ☒ BB Barbados
- ☒ BG Bulgaria
- ☒ BR Brazil
- ☒ BY Belarus
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China
- ☒ CU Cuba
- ☒ CZ Czech Republic
- ☒ DE Germany
- ☒ DK Denmark
- ☒ EE Estonia
- ☒ ES Spain
- ☒ FI Finland
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia
- ☒ GH Ghana
- ☒ GM Gambia
- ☒ HR Croatia
- ☒ HU Hungary
- ☒ ID Indonesia
- ☒ IL Israel
- ☒ IN India
- ☒ IS Iceland
- ☒ JP Japan
- ☒ KE Kenya
- ☒ KG Kyrgyzstan
- ☒ KP Democratic People's Republic of Korea
- ☒ KR Republic of Korea
- ☒ KZ Kazakhstan
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova
- ☒ MG Madagascar
- ☒ MK The former Yugoslav Republic of Macedonia
- ☒ MN Mongolia
- ☒ MW Malawi
- ☒ MX Mexico
- ☒ NO Norway
- ☒ NZ New Zealand
- ☒ PL Poland
- ☒ PT Portugal
- ☒ RO Romania
- ☒ RU Russian Federation
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia
- ☒ SK Slovakia
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan
- ☒ TM Turkmenistan
- ☒ TR Turkey
- ☒ TT Trinidad and Tobago
- ☒ UA Ukraine
- ☒ UG Uganda
- ☒ US United States of America
- ☒ UZ Uzbekistan
- ☒ VN Viet Nam
- ☒ YU Yugoslavia
- ☒ ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ AE United Arab Emirates
- ☒ ZA South Africa
- ☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box IV:

GOLDIN, Douglas Michael; ELLIS-JONES, Patrick George Armine; BARLOW, Roy James; SENIOR, Alan Murray; BENTHAM, Stephen; AYERS, Martyn Lewis Stanley; WOODS, Geoffrey Corlett; CRESSWELL, Thomas Anthony; SEXTON, Jane Helen; NICHOLLS, Michael John; MARSHALL Monica Anne; WEBB, Andrew John; KEEN, Celia Mary; PRICE, Nigel John King; IRVINE, Jonquil Claire; LEEMING, John Gerard; DUCKWORTH, Timothy John; MCCLUSKIE, Gail Wilson; WRIGHT, Simon Mark; CURWEN, Julian Charles Barton; CLEEVE, James Harold Findlay; SMITH, Samuel Leonard; BENSON, John Everett, CAMPBELL Patrick John; MERRYWEATHER, Colin Henry; DUCKETT, Anthony Joseph; BENTHAM, Andrew; and ROQUES, Sarah Elizabeth; SRINIVASAN, Ravi Chandran; FAULKNER, Charlotte Waveney and TYSON, Robin Edward of: J.A. KEMP & CO., 14 South Square, Gray's Inn, London, WC1R 5LX, United Kingdom.

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 6 MAY 1998 (06.05.98)	9809666.2	GB		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / EPO

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	: 5
description (excluding sequence listing part)	: 40
claims	: 4
abstract	: 1
drawings	: 14
sequence listing part of description	:
Total number of sheets	: 64

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: PF 23/77

Figure of the drawings which should accompany the abstract: 1

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

IRVINE, Jonquil Claire

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

EH459274014GS

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

From the INTERNATIONAL BUREAU

To:

IRVINE, Jonquil, Claire
J. A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

20 JUN

Action

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
22 June 1999 (22.06.99)	
Applicant's or agent's file reference	**International application No.**
N.74383A JCI	PCT/GB99/01413

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

ISIS INNOVATION LIMITED (for all designated States except US)
BROWNLEE, George, Gow et al (for US)

International filing date	:	06 May 1999 (06.05.99)
Priority date(s) claimed	:	06 May 1998 (06.05.98)
Date of receipt of the record copy by the International Bureau	:	01 June 1999 (01.06.99)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,SD,SL,SZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CU,CZ,DE,DK,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,US,UZ,VN,YU,ZA,ZW

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [ ] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

F. Gateau

Telephone No. (41-22) 338.83.38

JCI

PATENT COOPERATION TREATY

EH459274014US

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

IRVINE, Jonquil, Claire
J. A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

Date of mailing (day/month/year) 08 July 1999 (08.07.99)	
Applicant's or agent's file reference N.74383A JCI	**IMPORTANT NOTIFICATION**
International application No. PCT/GB99/01413	International filing date (day/month/year) 06 May 1999 (06.05.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 06 May 1998 (06.05.98)
Applicant ISIS INNOVATION LIMITED et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
06 May 1998 (06.05.98)	9809666.2	GB	15 June 1999 (15.06.99)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No. (41-22) 338.83.38

002721890

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EP

EH459274014US

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: N.74383A - JCI

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/GB99/01413	6 MAY 1999	6 MAY 1998

Title of invention: MODIFIED VIRUSES

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ISIS INNOVATION LIMITED
2 South Parks Road
Oxford, OX1 3UB
United Kingdom

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BROWNLEE, George Gow
Sir William Dunn School of Pathology
University of Oxford
Oxford, OX1 3RE
United Kingdom

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

FODOR, Ervin
Sir William Dunn School of Pathology
University of Oxford
Oxford, OX1 3RE

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
SK	GB

[X] Further applicants are indicated on a continuation sheet.

International application No.
PCT/GB99/01413

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PALESE, Peter
Department of Microbiology
Mount Sinai School of Medicine
1, Gustave L. Levy Place
New York 10029
United States of America

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
US	US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GARCIA-SASTRE, Adolfo
Department of Microbiology
Mount Sinai School of Medicine
1, Gustave L. Levy Place
New York 10029
United States of America

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
ES	US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/GB99/01413

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

IRVINE, Jonquil Claire
J.A. KEMP & CO.,
14 South Square,
London, WC1R 5LX,
United Kingdom.

Telephone No.: +44 171 405 3292

Facsimile No.: +44 171 242 8932

Teleprinter No.: 23676

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[X] the international application as originally filed

the description [ ] as originally filed
[ ] as amended under Article 34

the claims [ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: ENGLISH

[X] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[X] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

See Notes to the demand form

Sheet No. 4

International application No.
PCT/GB99/01413

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	1 sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [X] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

IRVINE, Jonquil Claire

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

PATENT COOPERATION TREATY

EH 459274014GS

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
J.A. KEMP & CO.
Attn. IRVINE, J.
14 South Square
Gray's Inn
London WC1R 5LX
UNITED KINGDOM

J. A. KEMP & Co
REC'D - 1 NOV 1999
Action by.....

	Date of mailing *(day/month/year)* 29/10/1999
Applicant's or agent's file reference N.74383A JCI	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/GB 99/01413	International filing date *(day/month/year)* 06/05/1999
Applicant ISIS INNOVATION LIMITED et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andria Overbeeke-Siepkes

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

ATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
N.74383A JCI		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 01413	06/05/1999	06/05/1998
Applicant		
ISIS INNOVATION LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

ATTENUATED INFLUENZA VIRUSES

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[ ] None of the figures.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/01413

Box I Observations where certain claims were found unsearchable (Continuation of it m 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 26
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INT ATIONAL SEARCH REPORT

[redacted]national Application No
PCT/GB 99/01413

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/44 C12N7/01 C12N15/86 A61K39/145

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 704 533 A (HOBOM G ET AL.; BAYER AG) 3 April 1996 (1996-04-03) page 4, line 25 -page 5, line 35; figures 1,2 page 2, line 40 - line 49 ---	1-27
X	BERGMANN M AND MUSTER T: "The relative amount of influenza A virus segment present in the viral particle is not affected by a reduction in replication of that segment" JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 12, December 1995 (1995-12), pages 3211-3215, XP002112523 READING GB cited in the application page 3214; figure 1 --- -/--	1-9, 13-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 October 1999	29/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL- 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KIM H-J ET AL: "Mutational analysis of the RNA-fork model for the influenza A virus vRNA promoter in vivo" JOURNAL OF GENERAL VIROLOGY, vol. 78, no. 2, February 1997 (1997-02), pages 353-357, XP002112524 READING GB cited in the application figures 1,2 ---	1-9
X,P	FODOR E ET AL.: "Attenuation of influenza A virus mRNA levels by promoter mutations" JOURNAL OF VIROLOGY, vol. 72, no. 8, August 1998 (1998-08), pages 6283-6290, XP002112525 AMERICAN SOCIETY FOR MICROBIOLOGY US the whole document -----	1-17, 22-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01413

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0704533 A	03-04-1996	AU 3607695 A	26-04-1996
		WO 9610641 A	11-04-1996
		EP 0783586 A	16-07-1997
		FI 971272 A	26-05-1997
		NZ 293600 A	28-01-1999

EH459274014US

JC

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
IRVINE, Jonquil Claire
J.A. KEMP & CO
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

J. A. KEMP & CO
- 2 DEC 1999

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*	30. 11. 99
Applicant's or agent's file reference: N.74383A JCI	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB 99/01413	06/05/1999	06/05/1998

Applicant

ISIS INNOVATION LIMITED et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

22/11/1999

2. This date of receipt is:

☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).

☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).

☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

☐ *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	AITKEN J M Tel. (+49-89) 2399-2735

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

PCT/GB99/01413

EH 45 92 74 01 4 US

PATENT COOPERATION TREATY

JCI

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

IRVINE, Jonquil, Claire
J. A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

Date of mailing (day/month/year) 17 December 1999 (17.12.99)	
Applicant's or agent's file reference N.74383A JCI	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB99/01413	06 May 1999 (06.05.99)	06 May 1998 (06.05.98)

Applicant
ISIS INNOVATION LIMITED et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,
   ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,
   TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Juan Cruz

Telephone No. (41-22) 338.83.38

WO 99/57284
PCT/GB99/01413

PATENT COOPERATION TREATY

EH459274014GB

From the INTERNATIONAL BUREAU

PCT

To:
IRVINE, Jonquil, Claire
J. A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year)		
11 November 1999 (11.11.99)		
Applicant's or agent's file reference N.74383A JCI	**IMPORTANT NOTICE**	
International application No. PCT/GB99/01413	**International filing date** (day/month/year) 06 May 1999 (06.05.99)	**Priority date** (day/month/year) 06 May 1998 (06.05.98)
Applicant ISIS INNOVATION LIMITED et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,
   HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,
   SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   11 November 1999 (11.11.99) under No. WO 99/57284

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

EH4592 #740145

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

IRVINE, Jonquil Claire
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

J. A. KEMP & Co
23 AUG 2000
Action by

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	18.08.00
Applicant's or agent's file reference: N.74383A JCI	IMPORTANT NOTIFICATION
International application No.: PCT/GB99/01413	International filing date *(day/month/year)*: 06/05/1999
Priority date *(day/month/year)*: 06/05/1998	
Applicant: ISIS INNOVATION LIMITED et al.	

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/ European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
N.74383A JCI		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/01413	06/05/1999	06/05/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/44		
Applicant		
ISIS INNOVATION LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/11/1999	18.08.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Steffen, P Telephone No. +49 89 2399 7307

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/01413

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-4,6-40	as originally filed			
5	as received on	19/05/2000	with letter of	17/05/2000

Claims, No.:

7-27	as originally filed			
1-6	as received on	19/05/2000	with letter of	17/05/2000

Drawings, sheets:

1/9-9/9	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

see separate sheet

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6,7,10,11,19-21,23-25,27
	No:	Claims	1-5,8,9,12-18,22,26
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	26,27 (see separate sheet)

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item I
Basis of the report

For the reasons as set out below, the amendments, in claims 1 and in the description on page 5, filed on 19.05.2000, have no basis in the description as originally filed and therefore introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

First is the replacement of the term "thereof" by "of said protein" is not supported by the description as originally filed. This is because no basis can be found in the application as originally filed for the functional modifications of an influenza viral protein appearing in new claim 1 and in the amended description on page 5 (please refer also to point VIII of the present report).

Second is the introduction of the term "is a non-chimeric duplex region, but" not supported by the description as originally filed and therefore not allowable under the terms of article 34(2)(b) PCT. The only reference which is made in the description to the term "chimeric" is with relation to the description of the prior art (e.g. D1, see description , page 1, last paragraph). Moreover is the document D1 not only accidentally anticipating the novelty of original claim 1, but is considered as being relevant prior art to both the questions of novelty and inventive step (see point V. of the present communication). Therefore the term "is a non-chimeric duplex region, but" is intended to disclaim the content of prior art D1 from the scope of claim 1. This in itself is not allowable. Furthermore, does a basis for this disclaimer not exist in the description as originally filed, since on page 5, lines 16-18 only "**native** influenza virus vRNA duplex region derived from..." are excluded from the "(mutated) duplex region" as referred to in claim 1. However the mutated duplex regions of D1 (D1, page 3212) cannot be considered as "native", since they are "mutant" (D1, page 3212, "We have now constructed to new mutant influenza A viruses, NA/X and NA/Y,... and Fig. 1).

In conclusion, due to the unallowed amendments filed, the present report is established on the application as originally filed.

Re Item IV

Lack of unity of invention

The present application refers to life attenuated influenza viruses, carrying different mutations in the 5'-3' terminal non-coding sequences of a viral genomic RNA segment, which allow for diminished expression of an influenza viral protein coding sequence and which cause an attenuated phenotype in mice. As will be detailed in point V of the present communication, the general common concept of invention e.g. mutations in the 5'-3' terminal non-coding sequences which cause diminished expression of an influenza viral protein coding sequence and an attenuated phenotype in mice, is not novel and inventive under the provisions of articles 33(2) and 33(3) PCT. Consequently, the present application lacks unity of invention under rule 68.1 PCT, because the different mutations in the 5'-3' terminal non-coding sequences are no longer linked to a common concept of invention by means of a special technical feature. The following inventions are found:

1. claims 1-5 and claims 8-27, all partly and claim 6 complete: with reference to attenuated influenza virus by introducing specifically the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus in the 5'-3' terminal non-coding sequences to diminish expression of an influenza viral protein coding sequence and attenuating the phenotype

2. claims 1-5 and claims 8-27, all partly and claim 7 complete: with reference to attenuated influenza virus by introducing specifically the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus as well as the U-G mutation at position 10 of the 3' terminus and the A-C mutation at position 11' at the 5' terminus in the 5'-3' terminal non-coding sequences to diminish expression of an influenza viral protein coding sequence and attenuating the phenotype.

Under the provisions of rule 68.1 PCT, examination is carried out on all parts of the application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: BERGMANN M AND MUSTER T: 'The relative amount of influenza A virus segment present in the viral particle is not affected by a reduction in replication of that segment' JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 12, December 1995 (1995-12), pages 3211-3215.

D2: EP-A-0 704 533 (HOBOM G ET AL.; BAYER AG) 3 April 1996 (1996-04-03)

The subject-matter of claims 6, 7, 10, 11, 19-21, 23-25 and 27 is not disclosed by the prior art on file and therefore meets the requirements of article 33(2) PCT.

Claims 1-5, 8, 9, 12-18, 22 and 26 lack novelty under article 33(2) PCT for the following reasons.

Novelty of claims 1-5 is anticipated by D1 (NA/Y mutant influenza A virus; abstract and page 3212, left column including figure 1). The parameters as defined in claim 4 (3-4 log reduction in plaque titre on MDCK cells, compared to wild type virus) are not specifically disclosed as such in D1, but at present it cannot be excluded that the mutant virus of D1 meets also with this requirement, especially as it meets with the requirements of claim 3 (one log reduction in plaque titre on MDBK cells, compared to wild type virus, D1 page 3214, left column, two last paragraphs). The virus of claims 8 and 9 and similarly the subject-matter of claims 12-16 and 22 is disclosed in D1 (NA/Y mutant influenza A virus; abstract and page 3212, left column including figure 1). The *ex-vivo* cell of claim 17 is also disclosed in D1 (page 3213, left column). Novelty of the vaccine and method of stimulating an immune response of claims 18 and 26 is anticipated in D1 (page 3214, left column, last paragraph and right column first and last paragraph, and table 1). It has to be noted here, that albeit in D1 an immune reaction was not monitored in mice after injection of the NA/Y mutant influenza A virus, it is evident that an immune response was triggered in those mice after injecting the attenuated virus.

In consequence, claims 1-5, 8, 9, 12-18, 22 and 26 lack novelty under article 33(2) PCT and also lack inventive activity under article 33(3) PCT.

More generally, claims 1-27 lack inventive activity under article 33(3) for the following

reasons.

The present application refers to life attenuated influenza viruses, carrying different mutations in the 5'-3' terminal non-coding sequences of a viral genomic RNA segment, which allow for diminished expression of an influenza viral protein coding sequence and which cause an attenuated phenotype in mice. Furthermore several modifications of these mutated viruses as well as uses and applications thereof are claimed.

The prior art D1 discloses such attenuated mutant influenza viruses and suggests their use as vaccines. D1 anticipates novelty of claims 1-5, 8, 9, 12-18, 22 and 26 and thus leaves it impossible to acknowledge inventive activity for these claims. Mutant influenza viruses comprising heterologous coding sequences and use thereof for pharmaceutical, vaccine or antigenic delivery purpose e.g. claims 10, 11, 19-21 and 27 are/is suggested in D2 (page 2, lines 40-50). Hence inventive activity can be acknowledged for these claims only in case they are based on novel and inventive mutant attenuated influenza viruses. The use of mutated attenuated influenza A virus as helper viruses for rescue purposes e.g. claims 23-25 is self-evident for the skilled person, once the properties of the attenuated phenotype are known. Likewise, inventive step for theses claims is only acknowledgeable, once they are based on claims which are novel and inventive.

The specific mutations which are disclosed in claims 6 and 7 (e.g. C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus and the C-A mutation at position 11 of the 3' terminus and the G-U mutation at position 12' at the 5' terminus combined with the U-G mutation at position 10 of the 3' terminus and the A-C mutation at position 11' at the 5' terminus, in the 5'-3' terminal non-coding sequences) represent in light of the prior art D1, alternative solutions to the provision of a life attenuated influenza virus. These specific mutations yield attenuated influenza viruses which do not show any other technical effects, with resect to viability and attenuation and likely also vaccination capacities than the NA/Y mutant influenza A virus of D1. In the absence of additional, unexpected technical effects, however, inventive activity cannot be acknowledged for claims 6 and 7, with respect to the specific mutations as referred to above.

In consequence, claims 1-27 lack inventive activity under article 33(3) PCT.

Claims 26 and 27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item VIII
Certain observations on the international application

The following objections under articles 5 and 6 PCT are raised.

Claim 1 is unclear in its wording with reference to "a functional modification thereof". It cannot be appreciated from the sentence construction if this modification refers either to "a mutated duplex region" or to "an influenza viral protein". Furthermore claim 1 is missing essential technical features to clearly define the subject-matter of said claim which leads to unclarity and which is contrary to the requirements of article 6 PCT in connection with rule 6.3(a) PCT. This is because of the term "at least one base pair substitution **such that** expression of said protein coding sequence...is reduced". Here the skilled person cannot contemplate which mutations have to be introduced into the 5'-3' duplex region so to reduce expression of said protein coding sequence and for attenuation of the virus. Clearly with the information given in claim 1, the skilled person is unable to carry out the invention, because not all mutations in the 5'-3' duplex region effectively lead to reduction in expression of the protein coding sequence and to attenuation of the virus as is outlined in the description for the D1 and D3 mutations/base pair substitutions (see example 4, page 22, lines 20-22 and more specifically example 5, page 23, lines 16-17 and example 13, pages 30-31, page 31, lines 8-10). Hence claim 1 is also prone to an objection under article 5 PCT.

The terms "functional modification thereof" and "functionally equivalent substitutions" in claims 1, 6, 7, 8 and 24 are unclear with respect to the nature of the modification/ substitution to be introduced and thus do not allow to suitably delimit the scope of these claims.

Claim 2 lacks clarity with respect to "a reduction in plaque titre". Since the amount of reduction is not specified, this term is prone to subjective interpretation, thus rendering the

scope of claim 2 unclear.

has also been shown that influenza A virus with the same base pair substitution is attenuated *in vivo* and can give rise to protective immunity against wild-type influenza A virus. Evidence suggests that such attenuation arises from reduced polyadenylation of the NA-specific mRNA. Base-pair substitution in the duplex region of a vRNA segment is thus proposed as a new general strategy for achieving attenuation of influenza viruses. Such base-pair substitution can be selected by application of known rescue systems for incorporating genetically-engineered influenza vRNA segments into viable influenza viruses as further discussed below.

In one aspect, the present invention thus provides an attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or functional modification of said protein, wherein said duplex region is a non-chimeric duplex region, but has at least one base-pair substitution such that expression of the said protein-coding sequence in cells infected by the said virus is reduced to give an attenuated phenotype.

Mutated duplex region of an influenza virus RNA genomic segment will be understood to exclude any native influenza virus vRNA duplex region derived from a vRNA of a wild-type influenza virus of a different type.

The term "cells" in this context may encompass human and/or animals cells *in vivo* normally infected by influenza viruses. For the purpose of selection of attenuated viruses of the invention, the same term will be understood to refer to cells of a single cell type or more than one type, e.g. cultured human or non-human animal cells of one or more than one type. They may be *in vivo* cells, e.g. cells of an animal model. Cultured cells which may prove useful in the selection of attenuated viruses of the invention *in vitro* include one or more of MDBK cells, Madin-Darby canine kidney (MDCK) cells and Vero (African green monkey kidney) cells.

While an attenuated virus of the invention may have a single base-pair substitution in the duplex non-coding region of a genomic segment, it will be appreciated that such a virus may have more than one such substitution, either on the same genomic segment or different genomic segments, e.g. 2 base pair substitutions in the same genomic segment duplex region. The duplex base-pair substitution(s)

CLAIMS

1. An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or a functional modification of said protein, wherein said duplex region is a non-chimeric duplex region, but has at least one base-pair substitution such that expression of said protein-coding sequence in cells infected by said virus is reduced to give an attenuated phenotype.

2. A virus as claimed in claim 1which exhibits a reduction in plaque titre compared to the parent wild-type virus on cells of one or more type selected from Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK) cells and Vero cells.

3. A virus as claimed in claim 2 which exhibits at least about one log reduction in plaque titre compared to the parent wild type virus on MDBK cells.

4. A virus as claimed in claim 2 or claim 3 which exhibits at least about 3 to 4 log reduction in plaque titre compared to the parent wild type virus on MDCK cells and Vero cells.

5. A virus as claimed in any one of claims 1 to 4 wherein said genomic nucleic acid segment is a mutated native influenza virus genomic RNA segment.

6. A virus as claimed in any one of claims 1 to 5 which is an attenuated influenza virus of type A, wherein said nucleic acid segment is a mutated influenza A virus genomic RNA segment having the mutation C to A at position 11 from the 3'-terminus of the native parent segment and the mutation G to U at position 12' from the 5'-terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an attenuating base-pair substitution in the non-coding duplex region.

PCT

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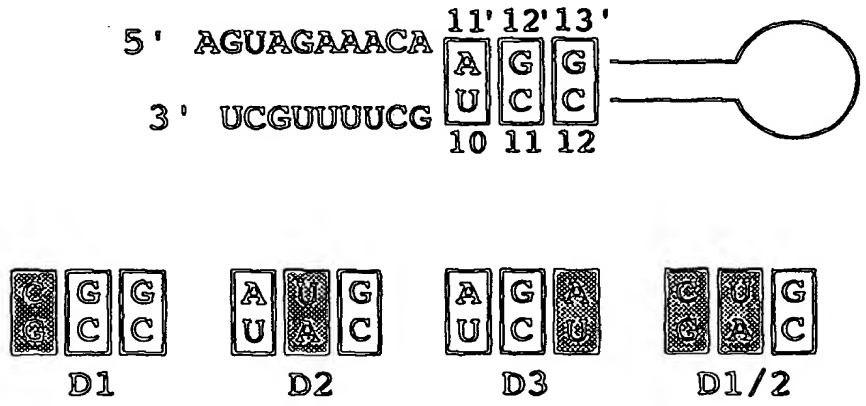
(54) Title: ATTENUATED INFLUENZA VIRUSES

(57) Abstract

An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non–coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably–linked to a protein coding sequence for an influenza viral protein or a functional modification thereof, wherein said duplex region has at least one base–pair substitution such that expression of said protein–coding sequence in cells infected by said virus is reduced to give an attenuated phenotype. The attenuated influenza virus can be used in a vaccine.



ATTENUATED INFLUENZA VIRUSES

The present invention relates to modified viruses, in particular attenuated influenza viruses which may be employed as an influenza virus vaccine. Modified viruses of the invention also include recombinant attenuated influenza viruses suitable for use as viral vectors for expression of heterologous sequences in target cells.

Influenza remains a constant worldwide threat to human health. While inactivated influenza virus vaccines have been available for many years, such vaccines provide only limited protection. Previous efforts to provide a safe, live attenuated influenza vaccine have focussed primarily on cold-adapted influenza viruses. Thus, attenuated influenza viruses have previously been obtained by extensively passaging influenza virus at low temperatures. As a result of adaptation to growth at low temperature, influenza viruses which have lost their ability to replicate at higher temperatures (about 39°C) are obtained. The replication of such cold-adapted (CA) viruses is only slightly restricted in the cooler upper respiratory tract, but highly restricted in the warmer lower respiratory tract, the major site of disease-associated pathology. Sequence comparisons between wild-type and CA influenza viruses have revealed both silent mutations and non-silent mutations leading to amino acid changes in the coding regions of several gene segments. Most amino acid changes were found to be the result of point mutations. The genetic instability of point mutations, and the level of immunogenicity of CA influenza viruses, remain as perceived potential problems in use of CA influenza viruses as vaccines for worldwide general use.

Another approach to obtaining attenuated influenza viruses which has been investigated is the construction of chimeric influenza viruses in which a non-coding region of an influenza virus genomic segment is substituted by a non-coding region from a genomic segment of an influenza virus of a different type. Such attenuated chimeric A/B influenza viruses are discussed, for example , in Muster *et al.*, Proc. Natl. Acad. Sci. USA (1991) 88, 5177-5181, Luo *et al.*, J. Virology (1992) 66, 4679-4685 and Bergmann and Muster, J. General Virology (1995) 76 3211-3215.

Three types of influenza virus are known designated as types A, B and C. Each of these types has many strains. The genome of an influenza virus is a segmented genome consisting of a number of negative sense RNAs (8 in the case of types A and B and 7 in the case of type C), which encode (in the case of type A) 10 polypeptides: the RNA-directed RNA polymerase proteins (PB1, PB2 and PA) and nucleoprotein (NP) which form the nucleocapsid, the matrix proteins (M1, M2), two surface glycoproteins which project from the lipoprotein envelope (hemagglutinin (HA) and neuraminidase (NA)) and the non-structural proteins NS1 and NS2. The majority of the genomic RNA segments are monocistronic. Thus, in the case of influenza virus of type A, 6 of the 8 genomic RNA segments are monocistronic and encode HA, NA, NP and the viral polymerase proteins, PB1, PB2 and PA.

During the replication cycle of an influenza virus, the viral genome (vRNA) is transcribed into mRNA and replicated into complementary RNA (cRNA) molecules, which in turn are used as templates for vRNA synthesis. These processes are known to be catalyzed by the viral polymerase complex consisting of three subunits formed by the PB1, PB2 and PA polypeptides. mRNA synthesis is initiated by capped RNA primers, which are cleaved from host cell mRNA by an endonuclease associated with the viral polymerase complex. The synthesis of mRNA is prematurely terminated at a run of uridines, in the case of an influenza A virus 16 or 17 nucleotides away from the 5' end of the vRNA template, and subsequently a poly(A) tail is added. On the other hand, cRNA synthesis is believed to be initiated in the absence of primer resulting in full-length precise copies of the vRNA segments. The nucleoprotein has been implicated as a switching factor, which acts as an antiterminator during cRNA synthesis.

Influenza vRNA segments may be prepared *in vitro* by transcription from plasmid DNA and mixed with viral polymerase proteins and nucleoprotein to form ribonucleoprotein complexes (RNPs) having all the components necessary for transcription and replication. Such RNPs can be incorporated into viable influenza virus particles in cell packaging systems, e.g. employing a helper virus.

The development of RNP reconstitution and transfection systems has permitted detailed characterization of the RNA signals in influenza A vRNAs

involved in the regulation of transcription initiation, termination, and polyadenylation (4, 20-22, 25, 32, 34). All these signals are known to reside in the terminal sequences of vRNA segments (19). The 5' and 3' ends contain 13 and 12 conserved nucleotides respectively, which have the ability to form a partially double-stranded panhandle/RNA-fork or corkscrew structure (6, 7, 13). Initial *in vitro* transcription studies with model RNA templates implied that vRNA and cRNA promoters were located exclusively in the 3' terminal sequences (25, 32) and that the panhandle had no apparent role in the initiation of transcription *in vitro*. However, detailed mutagenesis studies of the terminal sequences subsequently showed that the 5' end forms an integral part of the promoter. These findings were based on binding experiments of the RNA polymerase to the putative promoter RNA (7, 33) and, more importantly, on *in vitro* transcription studies with mutant model template RNAs (7, 8, 28). In addition, activation of the viral polymerase-associated endonuclease requires interaction of the polymerase complex with the 5' as well as the 3' terminal sequences of vRNA segments (11).

The postulated double-stranded region of the promoter of an influenza A vRNA segment is now recognised to consist of 5 to 8 base-pairs. The first 3 base-pairs, those formed by nucleotides 11' to 13' at the 5' end and nucleotides 10 to 12 at the 3' end, are strictly conserved among different vRNA segments of all influenza A viruses. Sequencing studies have shown that the 3' and 5' non-coding terminal sequences of influenza B and C vRNA segments are also highly conserved and show partial inverted complementarity (36, 37). Consequently, it is believed that the capability of base-pairing of nucleotides of the non-coding regions to form a panhandle structure is important for proper functioning of all influenza vRNAs. The term duplex region of an influenza vRNA segment as used hereinafter will be understood to refer to the region which is formed by such base-pairing.

Kim *et al.* (14) have previously used a choloramphenicol acetyltransferase (CAT) reporter gene construct in which negative sense CAT RNA is flanked by the non-coding sequences of an influenza A virus NS gene to determine the effect of mutations in the postulated duplex promoter region on CAT expression in Madin-Darby bovine kidney (MDBK) cells. Negative-sense CAT RNA constructs

were incorporated into RNP complexes, which were then used to transfect monolayers of MDBK cells infected with a helper influenza virus and CAT activity assayed. Using this model system, single mutations of the conserved residues at positions 11 and 12 of the 3' terminus and at positions 12' and 13' of the 5' terminus of the CAT gene construct were found to abolish or virtually abolish CAT activity. The introduction of second complementary mutations into such constructs so as to restore the capability for Watson-Crick base-pairing was found, however, to partially restore CAT activity. Thus, the constructs with the base-pair substitutions of U12-A13' for C12-G13' and A11-U12' for C11-G12' were found to express CAT at 31% and 22% respectively compared to the control construct with wild-type influenza A gene non-coding regions.

The same CAT reporter gene system was also used to investigate the effect of mutations of the U10-A11' base-pair. Single mutations, U10 to G10 and A11' to C11', significantly decreased CAT activity, but both mutants exhibited detectable activity. A combination of the two mutations to introduce a G10-C11' base-pair did not give improved CAT activity. It was therefore suggested that the properties of the base-pair at positions 10-11' might be different from those at positions 11-12' and 12-13'.

Such experiments merely test the effect of influenza vRNA duplex region mutations on the expression of a heterologous CAT reporter gene in cultured human cells. It is not possible to predict from such studies whether mutations which allow some CAT activity will, when incorporated into an influenza vRNA genomic fragment, permit rescue of that fragment into a viable virus. Equally, it is not possible to predict, even if such mutations give rise to viable virus, whether such viruses will be attenuated. Indeed, this is supported by the finding of the inventors that the base-pair substitution of C12-G13' by U12-A13' in the NA gene vRNA segment of an influenza A virus can be rescued into a viable influenza A virus which does not show significant attenuation on MDBK cells (see the Examples).

In contrast, it has now been established that substitution of A for C and U for G at position 11-12' in the duplex region of the NA-specific vRNA of an influenza A virus does lead to attenuation on MDBK cells and also other cell types in culture. It

has also been shown that influenza A virus with the same base pair substitution is attenuated *in vivo* and can give rise to protective immunity against wild-type influenza A virus. Evidence suggests that such attenuation arises from reduced polyadenylation of the NA-specific mRNA. Base-pair substitution in the duplex region of a vRNA segment is thus proposed as a new general strategy for achieving attenuation of influenza viruses. Such base-pair substitution can be selected by application of known rescue systems for incorporating genetically-engineered influenza vRNA segments into viable influenza viruses as further discussed below.

In one aspect, the present invention thus provides an attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of the said protein-coding sequence in cells infected by the said virus is reduced to give an attenuated phenotype.

Mutated duplex region of an influenza virus RNA genomic segment will be understood to exclude any native influenza virus vRNA duplex region derived from a vRNA of a wild-type influenza virus of a different type.

The term "cells" in this context may encompass human and/or animals cells *in vivo* normally infected by influenza viruses. For the purpose of selection of attenuated viruses of the invention, the same term will be understood to refer to cells of a single cell type or more than one type, e.g. cultured human or non-human animal cells of one or more than one type. They may be *in vivo* cells, e.g. cells of an animal model. Cultured cells which may prove useful in the selection of attenuated viruses of the invention *in vitro* include one or more of MDBK cells, Madin-Darby canine kidney (MDCK) cells and Vero (African green monkey kidney) cells.

While an attenuated virus of the invention may have a single base-pair substitution in the duplex non-coding region of a genomic segment, it will be appreciated that such a virus may have more than one such substitution, either on the same genomic segment or different genomic segments, e.g. 2 base pair substitutions in the same genomic segment duplex region. The duplex base-pair substitution(s)

will desirably result in some, e.g. at least about one log, reduction in plaque titre compared to the parent wild-type virus on MDBK cells. The duplex base-pair substitution(s) will desirably provide an attenuated virus exhibiting some, e.g. at least about one log, more preferably at least about 3 to 4 log, reduction of plaque titre on MDCK cells and Vero cells compared to the parent wild-type virus. An attenuated virus of the invention may, for example, exhibit as much as about 5 log reduction of plaque titre compared to the parent wild-type virus on Vero cells arising from the vRNA non-coding region base substitutions. Such an attenuated virus is exemplified by influenza A/WSN/33 having an NA-specific vRNA segment incorporating the base-pair substitution A11-U12' for C-G at position 11-12' of the duplex region and additionally having the base-pair substitution G10-C11' for U10-A11' (mutant D1/2 referred to in the examples). Other influenza A viruses incorporating the same base-pair substitutions, either in the NA-specific vRNA segment or a vRNA segment encoding another influenza virus protein, also exemplify the invention.

As indicated above, attenuated viruses of the invention also include influenza A/WSN/33 having the single base-pair substitution A11-U12' in the NA-specific vRNA segment (mutant D2 referred to in the examples) and other influenza A viruses having the same base-pair substitution in the NA-specific vRNA segment or another viral protein-encoding vRNA segment. Thus, in one embodiment the present invention provides an attenuated influenza virus of type A carrying a mutated influenza A virus genomic RNA segment having the mutation C to A at position 11 from the 3' terminus of the native parent segment and the mutation G to U at position 12' from the 5' terminus of the native parent segment, or functionally equivalent substitutions such as modified base substitutions at the same positions, so as to provide an attenuating base-pair substitution in the non-coding duplex region. Additionally, in a further embodiment, the present invention provides such an attenuated virus of type A which in the same vRNA segment has the mutation U to G at position 10 from the 3' terminus of the native parent segment and the mutation A to C at position 11' from the 5' terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an additional base-pair substitution in the non-coding duplex region. Such a virus may be a wild-type virus

which has been attenuated by introduction of one or more base-pair substitutions as above into the non-coding duplex region, or a recombinant attenuated virus carrying a heterologous coding sequence as further discussed below. Desirably, for example, the attenuating base-pair substitution(s) will be introduced into the genomic nucleic acid segment encoding NA or a functional modification of that surface glycoprotein.

Although the invention is further illustrated hereinafter with particular reference to influenza A/WSN/33, the invention is not confined to influenza viruses of the A-type. Functionally equivalent mutations to the D2 or D1/2 mutations, i.e. attenuating base-pair substitutions, in viruses of the B and C types may be analogously identified by reference to available sequence information and application of known rescue systems applicable to any genetically-engineered influenza vRNA segment suitable for providing the characteristic of attenuation to a complete influenza virus.

Thus, a further embodiment of the invention, is an influenza virus of type B carrying a mutated influenza B virus genomic RNA segment, e.g. NA-encoding segment, having an attenuating base-pair substitution in the non-coding duplex region at a functionally homologous position to the base-pair substitution in influenza A/WSN/33 designated above as D2. The invention also extends to influenza viruses of type C carrying such a base-pair substitution in a mutated influenza C virus genomic RNA segment, e.g. a mutated NA-encoding segment.

Brief Description of the Figures

Figure 1 is a representation of the conserved sequences of an influenza A virus vRNA in the panhandle/RNA-fork conformation (7, 13). Conserved base-pairs in the double-stranded region of the RNA-fork, involving both the 5' and 3' ends of the RNA segment, are boxed. Numbering of residues starts from the 3' end and from the 5' end. The 5' end numbers are distinguished by prime ('). Base-pairs in the conserved double-stranded region of the modified NA-encoding vRNA of the transfectant viruses designated D1, D2, D3 and D1/2 in the examples are shown. Changed base-pairs are highlighted.

Figure 2 shows growth curves of transfectant viruses on MDBK cells.

Confluent cells in 35 mm dishes were infected with wild-type influenza A/WSN/33 (wild-type; WT) virus, and with the transfectant D1, D2, D3 or D1/2 viruses at a multiplicity of infection (m.o.i.) of 0.01. At the indicated time points, infectious particles present in the media were titrated by plaque assay in MDBK cells. The presented values are averages from duplicate experiments.

Figure 3 shows the nucleotide sequence of the plasmid pT3NAm1 containing the full-length cDNA of the NA gene of influenza A/WSN/33 (positions 2412-3820) flanked by a unique BbsI restriction site at one end (position 2404) and a bacteriophage T3 RNA polymerase promoter at the other end (positions 3821-3836) in the background of the pUC19 cloning vector between the EcoR1 (position 2398) and Hind III (position 3837) restriction sites (9). This plasmid was employed to obtain the mutant versions of the NA-encoding vRNA of influenza A/WSN/33 present in the D1, D2, D3 and D1/2 viruses (see Example 1).

Figure 4 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with 10^3 plaque-forming units (pfu) (see Example 13).

Figure 5 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at 10^3 pfu.

Figure 6 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with $3x10^4$ pfu.

Figure 7 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at $3x10^4$ pfu.

Figure 8 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with 10^6 pfu.

Figure 9 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at 10^6 pfu.

Figure 10 shows viral titres (log pfu per ml) on lungs of mice at 3 days (left) and 6 days (right) post-infection , following intranasal infection with wild-type (WT) and D1, D2, D3 and D1/2 viruses at 10^3 pfu (see Example 14).

Figure 11 shows body weight of D2-immunised mice (3 dose levels: 10^6, $3x10^4$ and 10^3 pfu) following challenge with 10^6 pfu wild-type virus (see Example

15).

Figure 12 shows body weight of D1/2-immunised mice (3 dose levels: 10^6, $3x10^4$ and 10^3 pfu) following challenge with 10^6 pfu of wild-type virus.

A nucleic acid segment of a virus of the invention incorporating an attenuating base-pair substitution as discussed above, and DNAs capable of transcription to provide such a nucleic acid, also constitute additional aspects of the invention. A nucleic acid of the invention may preferably correspond to a mutated native influenza virus RNA genomic segment having an appropriate attenuating base-pair substitution in the non-coding duplex region. Such an RNA may have additional modifications, for example, one or more additional nucleotides added at the 3' and/or 5' terminus or internally which do not destroy function. It may be a chimeric RNA.

A DNA capable of transcription *in vitro* to provide an RNA nucleic acid segment of the invention may be initially constructed in a plasmid by application of conventional techniques and isolated from that plasmid by restriction endonuclease digestion. As illustrated by plasmid pT3NAm1 referred to above, for this purpose a cDNA of a native influenza virus vRNA segment may be inserted into a plasmid flanked by an appropriate promoter and a restriction endonuclease site. The cDNA may then be subjected to site-directed mutagenesis by, for example, PCR-directed mutagenesis employing appropriate mutagenic primers to provide a sequence encoding the desired mutated vRNA segment for transcription. Alternatively, a genomic nucleic acid segment of the invention may be synthesized.

For preparation of an attenuated virus of the invention, a genomic nucleic acid segment having at least one attenuating base-pair substitution as defined above may be complexed *in vitro* with influenza viral polymerase proteins and nucleoprotein to form a RNP complex. Such RNP complexes, which constitute a still further aspect of the present invention, may be prepared in conventional manner as previously employed for incorporation of genetically-engineered influenza virus RNA genomic segments into RNA complexes for viral rescue in cells (4, 5, 38). RNP complexes of the invention may be transfected into cultured cells, e.g. MDBK

cells, MDCK cells or Vero cells, again using conventional techniques. Methods commonly employed for this purpose include DEAE-dextran transfection and electroporation (19, 39).

In yet another aspect, the present invention provides a method of preparing an attenuated influenza virus of the invention which comprises providing in a host cell the genomic nucleic acid segments for said virus under conditions whereby said segments are packaged into a viral particle. For this purpose, the genomic nucleic acid segments may be provided in the host cell by plasmids. Alternatively, RNP complexes of the invention as hereinbefore described may be transfected into host cells that have previously been infected with an influenza helper virus to complement the RNP complexes and enable selection of the desired attenuated viral particles. A number of helper virus-based cellular rescue systems for particular influenza virus genes have previously been described and have been reviewed by Muster and García-Sastre (56). Such gene specific rescue systems are briefly summarized below.

Helper virus based influenza gene rescue systems

Helper based rescue systems have been reported allowing the genetic manipulation of influenza A vRNAs for NA and HA surface antigens, the non-structural proteins, NP, PB2 polymerase protein and the M proteins.

NA gene specific rescue system

The most commonly employed helper virus based influenza gene rescue system is limited to the NA of influenza A/WSN/33 virus (4, 5). This method is based on the observation that only influenza viruses with an NA gene from influenza A/WSN/33 are able to grow on MDBK cells in the absence of trypsin. In this rescue system, the helper virus is a reassortant containing seven gene segments from influenza A/WSN/33 and a NA gene from a virus other than influenza A/WSN/33. Generally A/WSN-HK, which has an NA gene from influenza A/HK/8/68, is used as the helper virus. In this system, the NA gene of influenza A/WSN/33 is transfected into cells infected with the helper virus. The virus is then selected by growing on MDBK cells in the absence of exogenous proteases.

NA genes can also be rescued by using a NA-deficient mutant virus as a helper virus. Such a helper virus requires exogenous neuraminidase to grow in tissue culture. The NA-gene is transfected into cells infected with the helper virus. The virus is then selected by growing on cells in the absence of neuraminidase (43).

5

NS gene specific rescue system

A temperature-sensitive influenza virus with a defect in the NS1 protein is used as the helper virus of a NS gene specific rescue system. The NS gene segment carries two overlapping genes coding for the NS1 and NS2 proteins. This rescue
10 system allows the rescue of a NS gene segment encoding an NS1 protein which has activity at the non-permissive temperature. In this system, the NS gene segment which is to be rescued is transfected into cells infected with the temperature-sensitive virus. The virus with the transfected NS gene segment is selected by growing the virus at the non-permissive temperature as described by Enami *et al.* (40).

15

PB2 gene specific rescue system

A virus with an avian influenza A virus PB2 gene can be used as the helper virus in a PB2 gene specific rescue system. The avian influenza A virus PB2 gene restricts the replication of the helper virus in mammalian cells. Therefore, this rescue
20 system can rescue a PB2 gene which allows replication of influenza virus in mammalian cells. The PB2 gene which is to be rescued is transfected into cells infected with the helper virus. The virus with the transfected PB2 gene is selected by growing the virus in mammalian cells. Subbarao *et al.* (41) have used such an avian influenza A virus PB2 gene based system to rescue the PB2 gene of wild-type
25 influenza A/Ann Arbor/6/60 virus.

M gene specific rescue system

An amantidine-sensitive influenza virus carrying an M gene of influenza A/equine/Miami/1/63 virus can be used as a helper virus of an M gene specific
30 rescue system. The rescue system allows the rescue of an M gene which confers amantidine resistance to a virus. In this system, the M gene which is to be rescued is

transfected into cells infected with the helper virus. The virus with the transfected M gene is selected by growing the virus in the presence of amantidine. Castrucci and Kawaoka (42) have used such an amantidine-sensitive M gene based system to rescue the M gene of influenza A/PR/8/34 virus.

Antibody-based rescue systems

These systems depend on the binding or non-binding of the transfectant virus to a particular antibody (5, 52). Such antibody is a neutralising antibody which binds to influenza virus and impairs its growth in tissue culture. The helper virus may, for example, carry a gene which encodes an influenza surface protein which displays the antibody epitope. This system can therefore be used to select for transfectant virus which does not carry such a gene, but which of course is viable. This type of rescue system thus allows the rescue of a gene encoding an influenza surface protein. The gene to be rescued is transfected into cells infected with the helper virus. The virus with the transfected gene is selected by growing the virus in the presence of the antibody. Such a system was used by Enami and Palese (5) to rescue a transfected synthetic HA segment.

NP gene specific rescue system

Li and coworkers (39) reported a reverse genetics system for the rescue of the influenza A virus nucleoprotein gene. In this system, a temperature-sensistive (ts) mutant ts56 is used as a helper virus. RNA complexes are reconstituted *in vivo* as described before (5) and are then introduced by electroporation into ts56 helper virus infected cells. Transfectant viruses with a rescued NP-encoding vRNA segment are selected at the non-permissive temperature by plaquing on MDBK cells.

Influenza B virus rescue system

Barclay and Palese (44) have additionally described the rescue of HA genes in an influenza B virus.

The preparation of an attenuated virus of the invention may alternatively be

achieved using the expression vector-based influenza gene rescue strategy developed by Pleschka *et al.* (45). In contrast to the RNP transfection system referred to above, this eliminates the need for purification of the viral NP and polymerase proteins which is required for *in vitro* reconstitution of RNP complexes. Expression vectors are co-transfected into host cells which will provide the NP and P proteins and also a genomic segment of the invention incorporating an attenuating base-pair mutation. In this case, RNP complexes of the invention are formed intracellularly. The cells may then be infected with an influenza helper virus as previously described to select for the required attenuated influenza virus .

An RNA complex of the invention may also be rescued in host cells into a viable attenuated virus by transfecting into the host cells additional complementing RNA complexes thereby eliminating the need for a helper virus. This may be achieved in accordance with the general rescue strategy for influenza virus genes more recently described by Enami (46). This strategy involves purifying RNPs from an appropriate influenza virus and treating the RNPs *in vitro* with RNase H in the presence of a cDNA which hybridizes to the influenza virus gene to be rescued. In this way specific digestion of that gene by the RNase H is achieved. The gene depleted RNPs are then co-transfected into cells with the RNP-complex containing the nucleic segment to provide the attenuating base-pair substitution. The cells are then overlaid with agar and transfectant attenuated viruses obtained by direct plaque formation. This strategy, unlike the above described helper virus-based gene rescue strategies, can be applied to any influenza gene from any influenza virus. It can thus be applied to obtain an attenuated virus or gene of the invention of any influenza type.

Since reversion of a base-pair mutation requires two specific mutations, attenuated influenza viruses of the invention are expected to be highly stable (see Example 12). Hence, such viruses may be particularly favoured for use as influenza virus vaccines.

As indicated above, a virus of the invention may additionally contain a heterologous coding sequence capable of being expressed in target cells. Such a heterologous coding sequence may encode an antigenic peptide or polypeptide

capable of stimulating an immune response (either an antibody response or a cell-mediated immune response) to a pathogenic agent. Representative examples of such pathogenic agents are viruses, e.g. other influenza viruses or non-influenza viruses such as HIV, bacteria, fungi, parasites, eg. malarial parasites, and disease-causing cells such as cancer cells.

Thus, in yet another aspect, the present invention provides a vaccine comprising a virus of the invention. Particularly preferred are such vaccines wherein the attenuated influenza virus acts as a combined vaccinating agent against more than one pathogenic agent, e.g. an influenza virus and a second pathogenic agent other than an influenza virus. Such vaccines may be formulated and administered in accordance with known methods for this purpose.

Thus in a still further aspect, the present invention provides a method of stimulating an immune response against an influenza virus, e.g. an influenza virus of Type A, either alone or together with stimulation of an immune response against one or more further pathogenic agents, which comprises administering in an immunising mode an attenuated influenza virus of the invention capable of inducing said immune response(s). Intranasal immunisation with an attenuated influenza virus of the invention may, for example, be preferred. Such immunisation may be carried out as illustrated by the immunisation studies with recombinant influenza viruses expressing an HIV-epitope reported by Muster *et al.* (49) and Ferko *et al.* (53) (see also Example 15). A suitable immunisation dose may be, for example, in the range of 10^3-10^9 pfu. Booster immunisations may be given following an initial immunisation with a virus having the same functional characteristics, but of a different subtype or type.

Methods for incorporating heterologous coding sequences into an influenza virus have previously been described, for example, in Published International Application WO91/03552 (Palese *et al.*) and are also reviewed by Muster and García-Sastre in Textbook of Influenza 1998 (56). The heterologous coding sequence may be on a genomic segment incorporating an attenuating base-pair substitution or on a different genomic segment. It may be carried by an additional nucleic acid segment also incorporating a gene for an influenza viral protein to

provide selection pressure. It has previously been reported, for example, that an influenza virus can be constructed carrying at least 9 different vRNA segments (40).

Use of attenuated recombinant influenza viruses of the invention as vectors to express foreign antigens for vaccinating purposes is an attractive therapeutic strategy since:

(i) Antibodies to the different subtypes show little cross-reactivity. One drawback with the use of a virus as a vaccine is that an immune response will be produced to the virus. It is often desired that one or more booster immunisations comprising the same antigen are given after the initial immunisation. However, the immune response to the virus reduces the effectiveness of subsequent immunisations with the same virus. Since antibodies to different influenza subtypes show little cross-reactivity, subsequent immunisations with an influenza virus of a different subtype but which expresses the same antigen should overcome this effect.

(ii) Influenza viruses have been shown to induce strong cellular and humoral responses.

(iii) Influenza viruses have been shown to induce strong mucosal responses. Intranasal immunisation with influenza virus has been shown to induce long lasting responses in genital and intestinal mucosa.

(iv) Influenza viruses are non-integrating and non-oncogenic.

(v) As previously noted above, attenuated influenza viruses of the invention can be anticipated to be attenuation stable.

For vaccinating purpose, a heterologous coding sequence may be provided in an attenuated virus of the invention encoding an antigen of a pathogenic agent or a modification thereof capable of stimulating an immune response. The heterologous coding sequence may be inserted into a viral gene to provide a fusion protein which retains the function of the parent viral protein. One site which has previously been found to tolerate insertions of foreign antigens (epitope grafting) is the antigenic B site of HA. Antigenic site B of that surface protein consists of an exposed loop structure located on top of the protein and is known to be highly immunogenic. Manipulation of the HA gene of an influenza virus to insert a viral epitope in the HA

protein B site has previously been reported (see again the studies of Muster *et al.* reported in 49 and the studies of Li *et al.* reported in 48). The same strategy has also previously been employed by Rodrigues *et al.* to express B-cell epitopes derived from a malaria parasite (50). Heterologous coding sequences for an antigenic polypeptide may also, for example, be preferably inserted into an influenza virus NA gene. Strategies for epitope grafting into influenza viral proteins have also previously been described, for example, in WO91/03552.

Epitope grafting of a foreign sequence into an influenza virus protein may result in a non-functional chimeric viral protein and make the rescue of a viable transfectant virus impossible. A different strategy for expressing foreign sequences by recombinant influenza viruses, which may be applied to attenuated viruses of the present invention, involves the engineering of gene segments containing an additional open reading frame. A recombinant genomic segment may be constructed which provides an internal ribosome entry site for a heterologous coding sequence. This approach has previously been used, for example by García-Sastre *et al.* to obtain an influenza virus vRNA segment which encodes both a truncated form of gp41 of HIV and NA (9). Alternatively, a heterologous coding sequence may be fused in frame to a viral protein coding sequence to encode a chimeric polyprotein capable of autoproteolytic protease cleavage to give the viral protein and a desired second polypeptide, e.g. a viral antigen. This strategy has been shown by Percy *et al.* to be suitable for expressing non-influenza proteins up to 200 amino acids in length (51).

It will be appreciated that a recombinant attenuated virus of the invention may be employed as a vehicle for expression of heterologous coding sequences in target cells for a variety of therapeutic purposes in addition to vaccination. Such a recombinant virus may, for example, have a genomic segment encoding any of the following:

- a cytokine such as an interferon or an interleukin,
- a toxin,
- a palliative capable of inhibiting a function of a pathogenic agent either directly or indirectly, e.g. a viral protease inhibitor
- an enzyme capable of converting a compound with little or no

cytotoxicity to a cytotoxic compound, e.g. a viral enzyme such as Herpes simplex thymidine kinase capable of phosphorylating purine and pyrimidine analogues to active toxic forms,

- an antisense sequence,
- a ribozyme.

Sequences encoding such agents may be incorporated into an attenuated influenza virus of the invention by any of the techniques previously referred to above in connection with providing attenuated viruses of the invention expressing foreign epitopes.

A heterologous coding sequence in an attenuated recombinant virus of the invention may be under the control of a tissue-specific and/or event-specific promoter. A recombinant virus of the present invention may be employed for gene therapy.

A recombinant virus of the invention may be administered directly or used to infect cells *ex vivo* which are then administered to a patient.

Thus, in still further aspects, the present invention provides a pharmaceutical composition comprising a recombinant virus of the invention in combination with a pharmaceutically acceptable carrier or diluent for delivery of a heterologous coding sequence to target cells. It also provides *ex vivo* cells infected by a virus of the invention and such cells hosting a recombinant influenza virus of the invention formulated for administration with a pharmaceutically acceptable carrier or diluent. In yet another aspect, the present invention provides a method of delivering a heterologous coding sequence to cells which comprises infecting said cells with an attenuated recombinant influenza virus of the invention carrying said sequence.

Viruses of the invention may also find use as a helper virus to rescue genes which can substitute for the gene(s) affected by the attenuating mutation(s) to provide viruses showing increased growth on a selected cell type. For this purpose, an attenuated virus will preferably be chosen which exhibits at least about a 3-4 log, preferably at least about a 5 log, reduction in growth compared to the corresponding wild-type virus on one or more cell types. Thus, in yet another embodiment, the present invention provides use of a virus of the invention as a helper virus to rescue

an influenza virus genomic nucleic acid segment in cells, wherein viruses produced containing said segment are selected on the basis of increased growth compared with the helper virus on cells of a selected type. For example, an influenza A virus of the invention having an attenuating base-pair substitution in the non-coding duplex region of its NA-encoding vRNA may be usefully employed to rescue an NA-encoding vRNA or functional modification thereof derived from a second influenza A virus. A typical protocol for this purpose will comprise the steps of:

1. infecting cells with the helper virus,
2. transfection of an RNP complex containing the gene(s) to be rescued into the helper virus infected cells, and
3. selection of rescued viruses, either on the same cell type or a different cell type on which the helper virus shows increased attenuation.

The cell type in step 3 will be chosen such that only viruses which have acquired the transfected gene(s) are expected to grow to high titre.

For example, the D1/2 mutant version of influenza A/WSN/33 referred to above is particularly favoured as a helper virus for use to rescue NA genes originating from other influenza viruses of the A-type. In this case, MDBK cells may, for example, be initially infected with the D1/2 helper virus and Vero cells preferably used for selection of viruses carrying an NA gene containing vRNA without an attenuating mutation. The D2 mutant derived from influenza A/WSN/33 may similarly be employed.

Influenza A/WSN/33 is known to exhibit in mice neurovirulence associated with the surface antigen NA (54). For this reason, the attenuated modified versions of that virus referred to above are not regarded as suitable for direct vaccine use. However, by using, for example, the D1/2 mutant as a helper virus as above, NA vRNAs may be obtained for site-directed mutagenesis to construct alternative attenuated influenza A viruses according to the invention more suitable for therapeutic, e.g. vaccine, use.

The following examples illustrate the invention.

Example 1

Introduction of mutations into the duplex region of the NA-encoding vRNA of an influenza virus of type A

In order to produce NA-encoding viral genomic RNA with mutations in the 5' and 3' non-coding regions, plasmids were constructed which contained the corresponding cDNA with the desired mutations.

The starting plasmid for site-directed mutagenesis was pT3NAm1 (see Figure 3) which, as previously noted above, contains the full length cDNA of the NA gene of influenza A/WSN/33 virus (positions 2412-3820) flanked by a unique BbsI restriction site at one end (position 2404) and a bacteriophage T3 RNA polymerase promoter at the other end (positions 3821-3836) in the background of the pUC19 cloning vector between the EcoR1 (position 2398) and Hind III (position 3837) restriction sites (9). Samples of influenza A/WSN/33 for preparation of the NA-encoding cDNA insert in plasmid pT3NAm1 are obtainable, for example, from the W.H.O. Collaborating Centre, Division of Virology, National Institute for Medical Research, London, U.K.

An alternative plasmid which may be employed to construct DNA templates for transcription of mutant NA-encoding vRNA segments of influenza A/WSN/33 is the pUC19-derived plasmid pT3NAv, whose construction is described in WO91/03552 (Palese, P. *et al.*). Plasmid pT3NAv also contains the full length cDNA of the NA gene of influenza A/WSN/33 flanked by a promoter specifically recognised by bacteriophage T3 RNA polymerase and a restriction endonuclease cleavage site.

PCR products were made using pT3NAm1 as a template and the following primers modified to provide mutations as specified in Fig. 1:
5'-CGGAATTCGAAGACGCAGCAAAAGCAGGAGTTTAAATGAATCC-3' (primer 1) and 5'-
CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTTTTTGAA C-3' (primer 2) (the residues at which mutations were introduced are underlined, e.g. for construction of the D1 mutant cDNA, in both primers 1 and 2 the first underlined A nucleotide was substituted by a C nucleotide). The PCR products were digested

with EcoRI and HindIII restriction enzymes and they were cloned into pT3NAm1 cut with the same enzymes. NA genes and the flanking sequences in the modified plasmids were sequenced with an automated sequencer (Applied Biosystems).

The following double-mutations were introduced into the NA gene of influenza A/WSN/33 virus: U-A→G-C (10-11') (mutant D1), C-G→A-U (11-12') (mutant D2), and C-G→U-A (12-13') (mutant D3) (Fig. 1). In addition, six NA genes with the corresponding single-mutations were constructed (U→G10, A→C11', C→A11, G→U12', C→U12, and G→A13').

Example 2

Production of and transfection of ribonucleoprotein (RNP) complexes.

Transfectant viruses were prepared as described by Enami and Palese (5). NA-specific RNP complexes were reconstituted *in vitro* and transfected into MDBK cells infected with A/WSN-HK helper virus (5).

Synthetic RNAs were obtained by T3 RNA polymerase transcription of modified pT3NAm1 plasmids linearized with BbsI restriction enzyme. RNAs were reconstituted into RNP complexes using RNA polymerase and NP protein isolated from influenza X-31 virus. Influenza X-31 virus is a reassortant of influenza A/HK/8/68 and A/PR/8/34 viruses and was supplied by Evans Biological, Ltd., Liverpool, England. The RNP complexes were transfected by the DEAE-dextran transfection method into MDBK cells infected with WSN-HK helper influenza virus grown in 10-day embryonated chicken eggs. The MDBK cells were grown in reinforced minimal essential medium. For subsequent experiments, influenza A/WSN/33 wild-type virus was also grown in MDBK cells in reinforced minimal essential medium. Rescued transfectant viruses were plaque purified three times in MDBK cells. A single plaque was used for preparing a stock virus for further analysis.

Example 3

Sequencing of the NA genes of transfectant viruses.

The presence of the mutations in the transfectants was confirmed by sequence

analysis of the 3' and 5' terminal sequences of the NA gene. Viral RNA for sequencing was isolated by phenol-chloroform extraction from transfectant viruses purified by centrifugation through a 30 % sucrose cushion. In some cases, total RNA isolated with RNAzol B (Tel-Test, Inc., Friendswood, TX) from infected cells was used. Sequences of the 5' end were obtained either by direct RNA sequencing or by 5' RACE. Direct sequencing of the 5' ends was performed using a primer complementary to nucleotide positions 1280 to 1299 (5'-TGGACTAGTGGGAGCATCAT-3') of the influenza A/WSN/33 NA gene and an RNA sequencing kit (United States Biochemical Corporation, Cleveland, OH) following the manufacturer's instructions. For 5' RACE, viral RNA was reverse transcribed using a primer complementary to nucleotide positions 879 to 898 (5'-GGGTGTCCTTCGACCAAAAC-3') of the influenza A/WSN/33 NA gene. The reverse transcription product was extended with terminal deoxynucleotidyl transferase (TdT) (Gibco BRL, Gaithersburg, MD) and amplified by PCR with the primer used for direct RNA sequencing (see above) and the 5' RACE abridged anchor primer (Gibco BRL). PCR products, cut with SpeI restriction enzyme, were cloned into the XbaI site of pUC18 and sequenced with a DNA sequencing kit (United States Biochemical). In order to sequence the 3' end of the NA gene of transfectant viruses, viral RNA was 3'-polyadenylated using poly(A) polymerase (Gibco BRL). The polyadenylated RNA was reverse transcribed using the primer 5'-GCGCAAGCTTCTAGATTTTTTTTTTTTT-3' and the cDNA was amplified by PCR with a primer containing nucleotides corresponding to positions 115 to 98 (5'-GCGCAAGCTTTATTGAGATTATATTTCC-3') of the influenza A/WSN/33 NA gene and the primer used for reverse transcription. PCR products digested with HindIII were cloned into pUC18 and sequenced with the DNA sequencing kit.

Transfection of all three NA genes with double-mutations resulted in rescue of transfectant viruses (D1, D2, and D3). On the other hand, only three out of the six single-mutant constructs were rescued, carrying mutations at positions 10, 11', and 13' (Fig. 1). In three attempts, none of the other three constructs (with mutations at positions 11, 12, and 12') was rescued.

Confirmation of mutations in the two single mutant transfectants at positions

10 and 11' was more difficult since they were unstable. Specifically, cloning of the 3' end of the NA vRNA of the U→G10 mutant resulted in one clone with mutant and two clones with wild-type sequences. Direct RNA sequencing of the 5' end of the NA-specific vRNA from purified A→C11' transfectant, following three plaque to plaque passages, revealed a wild-type sequence. However, when NA-specific vRNA from MDBK cells infected with the original plaque of this transfectant was sequenced, the presence of the mutation was confirmed. Thus it seems likely that the transfectant reverted to wild-type during the plaque purification steps. This interpretation is supported by the observation that the transfectant initially produced small plaques, but showed larger plaques upon passaging. Taken together, sequencing data of the single mutants showed that transfectant viruses with single mutations, at least those with mutations at positions 10 and 11', are unstable.

Example 4

Growth properties of the D1, D2, D1/2 and D3 mutants

D1, D2, and D3 were grown on MDBK cells. Confluent monolayers of MDBK cells were infected at low m.o.i. (0.01) and the amount of infectious virus released into the medium was assayed at different time points by plaque assay on MDBK cells (Fig. 2). The D2 transfectant virus showed approximately one log reduction in plaque titre compared to the wild-type virus. However, D1 and D3 transfectant viruses were not significantly affected by the mutations. Consistently, the plaque size of D2 was reduced, but both D1 and D3 viruses showed plaque sizes similar to that of the wild-type.

The growth properties were also investigated of mutant influenza A/WSN/33 having multiple double-mutations in the NA-specific vRNA. A construct incorporating double-mutations from both D1 and D2 transfectants was successfully rescued (D1/2) (Fig. 1) into infectious virus. The D1/2 transfectant was plaque purified three times and the presence of mutations was confirmed by sequencing. This virus showed similar reduction in plaque titres (Fig. 2) and plaque size on MDBK cells as the D2 transfectant. The effect of the D1/2 mutations on viral growth was more dramatic on MDCK and Vero cells where reductions of at least three to

four logs in plaque titres were observed (see Examples 10 and 11 below).

Example 5

Measurement of NA levels in transfectant viruses

The level of NA expressed by the viruses was determined to see if it corresponded to growth levels. Influenza A/WSN/33 and transfectant viruses were grown in MDBK cells and purified by 30 to 60% sucrose gradient ultracentrifugation. About 10 μg of viral proteins were denatured with 0.5% SDS and 1% β-mercaptoethanol at 100 °C for 10 minutes and digested with 400 u of PNGase F (New England Biolabs, Inc., Beverly, MA) for 20 h at 37 °C in a reaction buffer containing 50 mM sodium phosphate, pH 7.5, 1% NP-40, and 5 mM Pefabloc (Boehringer Mannheim Corporation, Indianapolis, IN). The PNGase F treatment removes N-linked carbohydrate chains from NA and HA. This gives a better resolution of the NA band which migrates closely to NP and HA on gels. Proteins were analyzed by 12% SDS-PAGE and staining with Coomassie Brilliant Blue.

Both D2 and D1/2 virions showed a dramatic reduction in NA content compared to that of the wild-type virus or the D1 and D3 transfectants.

In order to quantitate NA levels of the D2 and D1/2 viruses, neuraminidase activity was measured. About 2 μg, 0.5 μg, 0.125 μg, and 0.031 μg (4 fold dilutions) of proteins from purified virus were incubated for 10 minutes at 37 °C in 150 mM phosphate buffer, pH 6.0, 1 mM $CaCl_2$, containing 50 nmols of 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-NANA) as substrate in a total volume of 100 μl (27). Then 2 ml of stop buffer (0.5 M glycine/NaOH, pH 10.4) were added and the released 4-methylumbelliferone was determined by spectrofluorometry. 0.1 mM solution of 4-methylumbelliferone was used as a standard control. NA activity was expressed as nmoles of 4-methylumbelliferone released in 1 minute per μg of viral proteins.

NA activity associated with the wild-type virus was 2.18 nmol min^{-1} μg^{-1}. However, the transfectant viruses D2 and D1/2 exhibited only 0.24 and 0.25 nmol min^{-1} μg^{-1} activity, respectively. Thus, the transfectant viruses showed approximately a 10 fold reduction in NA activity compared to the wild-type virus which is in

agreement with the reduced NA levels observed in SDS-PAGE.

Example 6

NA-specific vRNA levels in purified transfectant viruses

Viral RNA from wild-type and transfectant viruses purified through a 30% sucrose cushion was extracted with phenol/chloroform. The viral RNAs purified from wild-type and transfectant viruses were analyzed by PAGE and the RNA segments were visualized by silver-staining. The NA segment was present in all transfectant viruses at levels comparable to that of the wild-type virus. In order to quantify NA-specific vRNA levels, a primer extension analysis was performed using vRNA extracted from purified viruses.

Primer extension analysis of NA and NS vRNA levels was performed as previously described (2). Briefly, 100 ng of viral RNA was transcribed with 200 u of SuperScript (Gibco BRL) for 1 h at 42 °C in the presence of 3×10^5 cpm of ^{32}P-labelled NA- and NS-specific primers. The NA-specific primer, 5'-GTGGCAATAACTAATCGGTCA-3', is complementary to nucleotides 1151 to 1171 of the NA vRNA. The NS-specific primer, 5'-GGGAACAATTAGGTCAGAAGT-3', is complementary to positions 695 to 715 of the NS vRNA. Primer extension reactions were stopped by adding an equal volume of 90% formamide and 10 mM EDTA followed by heating to 95 °C for 3 minutes. Extension products were analyzed on 5% polyacrylamide gels in the presence of 7 M urea and quantitated by phosphorimager analysis of dried gels (Molecular Dynamics).

The NS gene was used as an internal control. The amounts of NA-specific vRNA segments in the transfectant viruses were similar (±20%) to that of the wild-type virus in two experiments.

Example 7

NA-specific vRNA levels in cells infected with the D2 or D1/2 transfectant viruses.

MDBK cells were infected with wild-type or transfectant viruses at an m.o.i. of 2 and total RNA was isolated from cells at 3.0, 5.5, 8.0, and 10.5 h postinfection

with RNAzol B (Tel-Test). NA-specific vRNA levels in total RNA were measured by primer extension assay as described above in Example 6 using 5μg of total RNA. Cells infected with the D2 transfectant virus contained NA-specific vRNA levels similar (±10%) to those infected with the wild-type virus. Although cells infected with the D1/2 transfectant virus showed a 28 to 53% reduction in NA-specific vRNA levels (results obtained by phosphorimager analysis in two experiments at 5.5, 8.0, and 10.5 h postinfection), this decrease cannot account for the ten-fold reduction of NA protein levels.

Example 8

NA-specific mRNA and cRNA levels in cells infected with the D2 or D1/2 transfectant viruses.

Since NA-specific vRNA levels were not dramatically affected by the mutations in the D2 and D1/2 transfectant viruses, the 10 fold reduction in NA levels (see above) could result from a reduction in mRNA levels and/or from a defect in translation. In order to distinguish between these possibilities, the amounts of NA-specific mRNA in cells infected with D2 or D1/2 transfectant viruses were measured by using a primer extension assay. MDBK cells were infected at an m.o.i of 2 with wild-type or transfectant viruses and total RNA was isolated at 3.0, 4.5, 6.0, and 7.5 h postinfection.

Primer extension analysis of NA and HA mRNA and cRNA levels in total RNA from infected cells was performed under the same conditions as described in Example 6. The primer for NA-specific mRNA and cRNA, 5'-GCGCAAGCTTTATTGAGATTATATTTCC-3', contains 18 nucleotides (underlined) corresponding to positions 115 to 98 of the NA gene. The primer for the extension of HA-specific mRNA and cRNA, 5'-CATATTGTGTCTGCATCTGTAGCT-3', corresponds to positions 94 to 71 of the HA gene.

Since total RNA from infected cells contains both mRNA and cRNA, which differ only at their termini, signals for both species of RNAs were expected in the same primer extension assay. Due to the presence of a heterologous 10 to 15

nucleotides long capped primer at the 5' end of mRNA molecules, the signal for mRNA on gels appears as a multiple band containing DNA species of different sizes. On the other hand, the signal for cRNA appears as a single band, which is approximately 10 to 15 nucleotides shorter than the signal for mRNA.

NA-specific mRNA levels in cells infected with either D2 or D1/2 transfectant virus were below detection levels. NA-specific cRNA levels were apparently unaffected in these transfectant viruses. An additional band running slightly faster than the NA-specific cRNA band, detected in all samples, represents a nonspecific signal, since it was also detected in RNAs extracted from uninfected cells.

The observed attenuation of NA-specific mRNA levels in cells infected with the D2 transfectant is consistent with the previous findings of Kim *et al.* (14) that an A-U(11-12') base-pair mutation in the context of a vRNA-like CAT reporter gene resulted only in 22% reporter activity compared to a wild-type control. However, the G-C(10-11') and U-A(12-13') base-pair mutations, which had no effect on the expression levels of the neuraminidase of the D1 and D3 transfectants, resulted in only 20 and 31% activities, respectively, in a CAT reporter gene system (14). It is thus clear that base-pair mutations in the context of a CAT reporter gene system and a rescued native NA gene containing vRNA segment have different effects.

Example 9

In vitro transcription of NA-specific ribonucleoprotein complexes.

In theory, the reduction of mRNA levels observed as above could have been caused by a decrease in mRNA stability or by a decrease in mRNA synthesis. The interference with mRNA synthesis may occur at the point of initiation, e.g. capped RNA primer binding or endonuclease activity could be inhibited. Alternatively, termination or polyadenylation of viral mRNA could be affected. In order to distinguish between all these possibilities, *in vitro* transcription assays were performed.

Wild-type influenza A/WSN/33 virus, D2, and D1/2 transfectants were grown in MDBK cells and purified on a 30% sucrose cushion. Twelve 15 cm dishes were

used for each virus. The purified viruses were resuspended in 200 μl of PBS and disrupted by adding 50 μl of 5x disruption buffer (500 mM Tris-HCl [pH 7.4], 500 mM NaCl, 25 mM $MgCl_2$, 5 mM DTT, 25% glycerol, 2.5% NP-40, 2.5% Triton X-100, 50 mg ml^{-1} lysolecithin) and incubation at 37 °C for 30 min. The disrupted viruses were fractionated by centrifugation on a discontinuous glycerol gradient (70%, 50%, and 30%, 150 μl of each) in 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. The gradients were centrifuged for 4 h at 15 °C in 0.8 ml tubes at 45,000 rpm in a Beckman SW55 rotor with adaptors. Fractions collected from the bottom of the tubes were analyzed by 12% SDS-PAGE and those enriched in RNPs were used in transcription assays.

In vitro transcriptional activity was measured using globin mRNA as primer. Transcription reactions were performed by using 6 μl of RNPs in a total reaction volume of 20 μl containing 50 mM Tris-HCl (pH 7.8), 50 μM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.5 mM each GTP and CTP, 50 μM UTP, 0.1 μM [α-^{32}P] UTP (3,000 Ci $mmol^{-1}$), 20 u of RNase inhibitor (Boehringer Mannheim Corporation, Indianapolis, IN), 0.6 μg of rabbit globin mRNA (Gibco BRL). After incubation at 31 °C for 1.5 h, transcription products were extracted with phenol/chloroform and precipitated in the presence of 5 μg of carrier yeast RNA.

NA-specific transcription products were synthesized from both the wild-type and the transfectant RNPs. However, there was a significant difference in the pattern of the bands. The wild-type NA-specific transcription product appeared as a wide band corresponding to RNA species with poly(A) tails of different sizes. On the other hand, the NA-specific transcription products of both the D2 and D1/2 transfectants produced less diffuse bands, which implied that these products might not be polyadenylated. In order to characterize the transcription products, they were analyzed by oligo(dT)-cellulose chromatography.

The fractions depleted of poly(A)-containing molecules showed higher levels of NA-specific transcription products for the D2 and D1/2 transfectants, but lower levels for the wild-type control. On the other hand, fractions enriched in poly(A)-containing molecules showed lower levels of the NA-specific transcription products for the D2 and D1/2 transfectants, but higher levels for the wild-type virus.

This seems to confirm that there is a large proportion of NA-specific transcription products of the D2 and D1/2 transfectants which lack poly(A) tails.

It is thus proposed that the mutations in the NA-specific vRNA of D2 and D1/2 interfere with polyadenylation of mRNA transcripts. The observed low levels of mRNA in cells infected with these viruses is fully consistent with this conclusion, since non-polyadenylated capped transcripts are most likely rapidly degraded in the cell (30).

Example 10

Growth of transfectant viruses on MDCK cells.

MDCK cells in 96-well plates were infected with $5x10^4$ pfu and 10 times dilutions of wild-type influenza A/WSN/33 virus, or transfectant D1, D2, D3, and D1/2 viruses. Four wells were used for each virus. Infected cells were maintained in 100 μl of Dulbecco's minimal essential medium (DMEM) supplemented with 10% bovine serum albumin and 1 μg/ml of trypsin. After 72 h, 50 μl of the medium was tested for hemagglutination with 50 μl of 1.5% red blood cells and ID_{50} was calculated for each virus. ID_{50} is defined as the dose at which 50% of the medium of the infected cells gives a positive haemagglutination signal. It was found that the ID_{50} for the wild-type virus and the D1 transfectant was 5 pfu. On the other hand, the ID_{50} of the D3 transfectant was 20 times higher. The ID_{50} of the D2 and D1/2 transfectant was approximately 3000 times higher than that of the wild-type or the D1 transfectant.

Example 11

Growth of the D1/2 transfectant on Vero cells.

Confluent Vero cells in 35 mm dishes were infected at an m.o.i. of 0.01 with wild-type influenza A/WSN/33 virus or D1/2 transfectant in duplicates. Cells were maintained in DMEM supplemented with 2% FBS for 72 h and virus present in the medium was titrated by plaque assay on MDBK cells. The wild-type virus reached $5x10^7$ pfu/ml, but there was less than $5x10^2$ pfu/ml of infectious virus in the medium from the cells infected with the D1/2 transfectant.

Taken together, the data in Examples 4, 10 and 11 show that base-pair mutations in the double-stranded region of the promoter of an influenza A virus vRNA can lead to reduced growth of influenza virus in tissue culture. As noted above, the D2 and D1/2 transfectant viruses showed approximately one log reduction in growth in MDBK cells, while both the D1 and D3 viruses grew like the wild-type. A more dramatic reduction in growth was observed for the D2 and D1/2 viruses on MDCK and Vero cells. Interestingly, the D3 transfectant showed reduced growth on MDCK cells compared to the wild-type. Both D2 and D1/2 transfectants exhibited approximately four log reduction on MDCK cells, and the D1/2 transfectant 5 log reduction on Vero cells. Such results are indicative that influenza A viruses having the D2 and D1/2 mutations will exhibit effective attenuation *in vivo*.

Example 12

Passage of transfectant viruses and sequencing to determine the stability of the D1, D2 and D3 mutations

Stocks of D1, D2, and D3 transfectant viruses with confirmed double-mutations were plaqued on MDBK cells and individual plaques were passaged ten times on MDBK cells at a low m.o.i. After ten passages, the viruses were plaqued and single plaques were used to prepare virus stocks for sequencing. Stocks of passaged viruses were purified through a 30 % sucrose cushion and viral RNA was isolated by phenol-chloroform extraction. In order to sequence the 3' end of the NA gene, viral RNA was 3'-polyadenylated using poly(A) polymerase (Gibco BRL, Gaithersburg, MD). The polyadenylated RNA was reverse transcribed using the primer 5'-GCGCAAGCTTCTAGATTTTTTTTTTTTTT-3' and the cDNA was amplified by PCR with a primer containing nucleotides corresponding to positions 115 to 98 (5'-GCGCAAGCTTTATTGAGATTATATTTCC-3') of the influenza A/WSN/33 NA gene and the primer used for reverse transcription. PCR products digested with HindIII were cloned into pUC18 and sequenced with a DNA sequencing kit (United States Biochemical, Corporation, Cleveland, OH).

Three clones originating from three individually passaged plaques of the D1 transfectant showed the presence of the U→G10 mutation. All clones obtained from 5

individually passaged plaques of the D2 transfectant had the expected C→A11 mutation. In addition, two of the clones showed a U→C change at position 4 which is a natural variation observed among different influenza A virus isolates. In two of the clones, we have also found a U→C mutation at position 23 adjacent to the initiation codon for the neuraminidase which changes the second amino acid of NA from an asparagine to an aspartate. Only two of the clones obtained from the D3 transfectant showed the C→U12 mutation. The third clone had a wild-type sequence indicating that this base-pair mutation might not be stable. A reversion of A→G13' could result in a viable virus with a U-G(12-13') base-pair, which could then revert to the wild-type C-G(12-13') base-pair by a U→C12 change. Due to the presence of different residues such a reversion cannot occur at the other two studied base-pairs.

In summary, the mutations in the 3' end of D1 and D2 transfectants were preserved during ten passages. Preliminary data confirms the presence of the mutations also in the 5' end of the NA segment of the passaged transfectant viruses. It can be assumed that transfectant viruses with double-mutations should be stable since two specific mutations would have to occur simultaneously in order to revert to the wild-type sequence. It did not prove possible to rescue any transfectant viruses with C→A11 or G→U12' single mutations which suggests that such viruses might be severely impaired or not viable at all.

Example 13

Attenuation of D2 and D1/2 viruses in mice

Influenza A/WSN/33 wild-type and transfectant viruses D1, D2, D3 and D1/2 were grown at 37°C in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium. Plaque assays were performed on MDBK cells.

Groups of five female BALB/c mice were used for influenza virus infection at 6 to 12 weeks of age. Intranasal (i.n.) inoculations were performed in mice under ether anesthesia using 50μl of PBS containing 10^6, $3x10^4$ or 10^3 plaque forming units (pfu) of D1, D2, D3 or D1/2 virus. As controls, mice were infected with wild-type influenza A/WSN/33 virus using the same pfu of virus. This virus was rescued by ribonucleoprotein transfection of a wild-type NA gene as previously described by

Enami and Palese (4). Animals were monitored daily and sacrificed when observed in extremis. All procedures were in accord with NIH guidelines on care and use of laboratory animals. The results are shown in Figures 4 to 9.

All mice infected with wild-type virus developed signs of disease and died by day 15 post-infection. However, all mice infected with the D2 or D1/2 viruses survived. Only those D2 or D1/2 virus-infected animals lost weight which were infected with the high dose of virus (10^6 pfu); they lost 10 to 20% of body weight by day 3 post-infection, but they quickly recovered in the following days. The virulence of the D1 virus was indistinguishable from the virulence of wild-type virus in these experiments. The D3 virus showed a slightly attenuated phenotype in mice.

Example 14

Impaired replication of the D2 and D1/2 viruses in mouse lungs

Groups of 6 BALB/c mice were infected intranasally as above with 10^3 pfu of wild-type, D1, D2, D3 or D1/2 viruses. Three days post-infection, three mice per group were sacrificed, their lungs were extracted and homogenized in 2 ml of PBS, and virus titres were measured by plaque assay in MDBK cells. Six days post-infection, the rest of the mice were also sacrificed and viral titres were determined in their lungs by the same protocol. The results are shown in Fig. 10.

The wild-type and the D1 viruses grew to high titres in the lungs of the infected mice (approximately 10^6 and 10^7 pfu/ml at days 3 and 6 post-infection, respectively). Titres in the lungs of mice infected with the D3 virus were approximately one and a half logs lower. By contrast, viral titres were not detectable or very low (less than 10^3 pfu/ml) in the lungs of the D2 or D1/2 infected mice. The results demonstrate that replication of the D2 and D1/2 viruses is highly impaired in mouse lungs.

Example 15

Induction of protective immunity by D2 and D1/2 viruses

Sera from the groups of surviving mice which were intranasally infected with D2 or D1/2 virus as above was collected and pooled 3 weeks after infection. The

sera were treated with receptor destroying enzyme (Sigma) to eliminate unspecific inhibitors of influenza virus-mediated haemagglutination as previously described by Burnet and Stone (55). The haemagglutination inhibition (HI) titres were determined as the highest serum dilution that was able to neutralize the haemagglutination activity of a preparation of influenza A/WSN/33 virus with an HA titre of 8. In these assays, 0.5% chicken red blood cells were used.

All pools of sera which were tested were found to contain antibodies against influenza A/WSN/33 virus with HI activity. HI titres were higher in the animals immunized with the higher virus doses (see Table 1 below).

In addition, all mice which were intranasally infected with D2 or D1/2 virus were observed to be protected against death and disease (as measured by body weight loss) when challenged with a lethal infection dose (more than 1000 LD_{50}s) of wild-type A/WSN/33 virus (see Table 1 and Figures 11 and 12).

Table 1

Protection against wild-type influenza virus infection

in mice immunized with D2 and D1/2 viruses

Immunizing virus	Immunizing dose	HI titres	Challenge: 10^6 pfu of wild-type virus Number of survivors
D2	10^6 pfu	352	5/5
	3 x 10^4 pfu	160	5/5
	10^3 pfu	24	5/5
D1/2	10^6 pfu	160	5/5
	3 x 10^4 pfu	44	5/5
	10^3 pfu	72	5/5

Example 16

Use of the D1/2 transfectant virus as a helper virus to rescue NA genes

As noted above, the D1/2 transfectant virus showed approximately 5 log reduction in growth on Vero cells compared to wild-type influenza A/WSN/33. It can therefore be employed to provide an alternative rescue system for rescue of

NA-encoding vRNA segments of influenza A viruses. An appropriate protocol for this consists of the following steps:

1. infection of MDBK cells with D1/2 helper virus;
2. treatment of the infected MDBK cells with DEAE-dextran/DMSO transfection reagent;
3. transfection of a synthetic NA ribonucleoprotein complex into D1/2 helper virus infected and DEAE-dextran/DMSO-treated MDBK cells; and
4. selection of rescued viruses on Vero cells.

Only viruses which acquire the transfected NA gene grow to high titre on Vero cells.

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CLAIMS

1. An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or a functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of said protein-coding sequence in cells infected by said virus is reduced to give an attenuated phenotype.

2. A virus as claimed in claim 1which exhibits a reduction in plaque titre compared to the parent wild-type virus on cells of one or more type selected from Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK) cells and Vero cells.

3. A virus as claimed in claim 2 which exhibits at least about one log reduction in plaque titre compared to the parent wild type virus on MDBK cells.

4. A virus as claimed in claim 2 or claim 3 which exhibits at least about 3 to 4 log reduction in plaque titre compared to the parent wild type virus on MDCK cells and Vero cells.

5. A virus as claimed in any one of claims 1 to 4 wherein said genomic nucleic acid segment is a mutated native influenza virus genomic RNA segment.

6. A virus as claimed in any one of claims 1 to 5 which is an attenuated influenza virus of type A, wherein said nucleic acid segment is a mutated influenza A virus genomic RNA segment having the mutation C to A at position 11 from the 3'-terminus of the native parent segment and the mutation G to U at position 12' from the 5'-terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an attenuating base-pair substitution in the non-coding duplex region.

7. A virus as claimed in claim 6 wherein said nucleic acid segment also has the mutation U to G at position 10 from the 3' terminus of the native parent segment and the mutation A to C at position 11' from the 5' terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an additional base-pair substitution in the non-coding duplex region.

8. A virus as claimed in claim 6 or claim 7 wherein said nucleic acid segment encodes neuraminidase (NA) or a functional modification thereof.

9. A virus as claimed in any one of claims 1 to 8 which is a wild-type virus which has been attenuated by said base-pair substitution(s).

10. A virus as claimed in any one of claims 1 to 8 which additionally comprises a heterologous coding sequence capable of being expressed in target cells.

11. A virus as claimed in claim 10 wherein said heterologous coding sequence encodes an antigenic peptide or polypeptide capable of stimulating an immune response to a pathogenic agent.

12. A virus as claimed in claim 9 which is attenuated influenza A/WSN/33 having a NA-encoding nucleic acid segment as defined in claim 8.

13. A nucleic acid as defined in claim 1 or any one of claims 5 to 8.

14. A DNA capable of transcription to provide a nucleic acid according to claim 13.

15. A plasmid containing a DNA as claimed in claim 14.

16. A ribonucleoprotein (RNP) complex wherein a nucleic acid as claimed in claim 13 is complexed with polymerase proteins and nucleoprotein of an influenza

virus for use in preparing an attenuated virus as claimed in any one of claims 1 to 12.

17. An *ex vivo* cell infected by a virus as claimed in any one of claims 1 to 12.

18. A vaccine comprising a virus as claimed in any one of claims 1 to 11.

19. A vaccine as claimed in claim 18 which comprises a virus as claimed in claim 11 and which is capable of stimulating an immune response to an influenza virus and a second pathogenic agent other than an influenza virus.

20. A pharmaceutical composition comprising a virus as claimed in claim 10 in combination with a pharmaceutically acceptable carrier or diluent for delivery of said heterologous coding sequence to target cells.

21. A pharmaceutical composition comprising cells infected with a virus according to claim 10 or claim 11 in combination with a pharmaceutically acceptable carrier or diluent.

22. A method of preparing a virus according to any one of claims 1 to 12 which comprises providing in a host cell the genomic nucleic acid segments for said virus under conditions whereby said segments are packaged into a viral particle.

23. Use of a virus as claimed in any one of claims 1 to 12 as a helper virus to rescue an influenza virus genomic nucleic acid segment in cells, wherein viruses produced containing said nucleic acid segment are selected on the basis of increased growth compared with the helper virus on cells of a selected type.

24. Use of an influenza A virus as claimed in claim 8 as a helper virus in accordance with claim 23 to rescue an NA-encoding influenza A virus genomic nucleic acid segment or a functional modification thereof.

25. Use as claimed in claim 24 of attenuated influenza A/WSN/33 having mutations as defined in claim 7 in the NA-encoding genomic RNA segment, wherein selection of viruses carrying the nucleic acid segment to be rescued is carried out on Vero cells.

26. A method of stimulating an immune response against an influenza virus, optionally together with stimulation of an immune response against one or more further pathogenic agents, which comprises administering in an immunising mode an attenuated influenza virus as claimed in any one of claims 1 to 11.

27. A method of delivering a heterologous coding sequence to cells which comprises infecting said cells with a virus according to claim 10 carrying said sequence.

Fig.1.

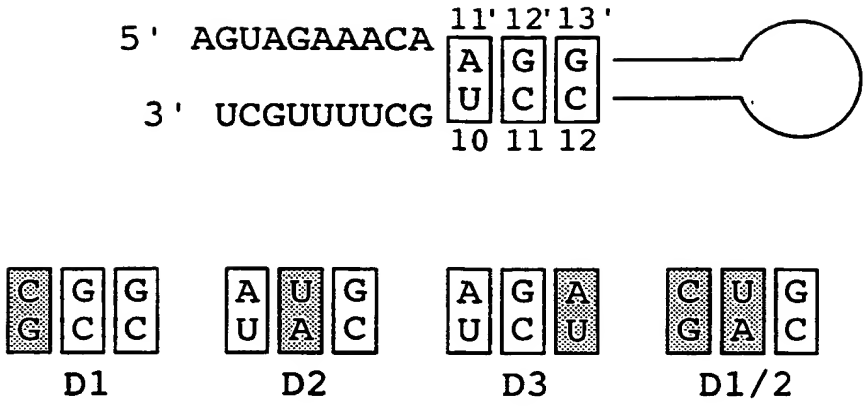

5' AGUAGAAACA
3' UCGUUUUCG
11' 12' 13'
A U G C G C
10 11 12
C G G C G C
D1
A U U A G C
D2
A U G C A U
D3
C G U A G C
D1/2

Fig.2.

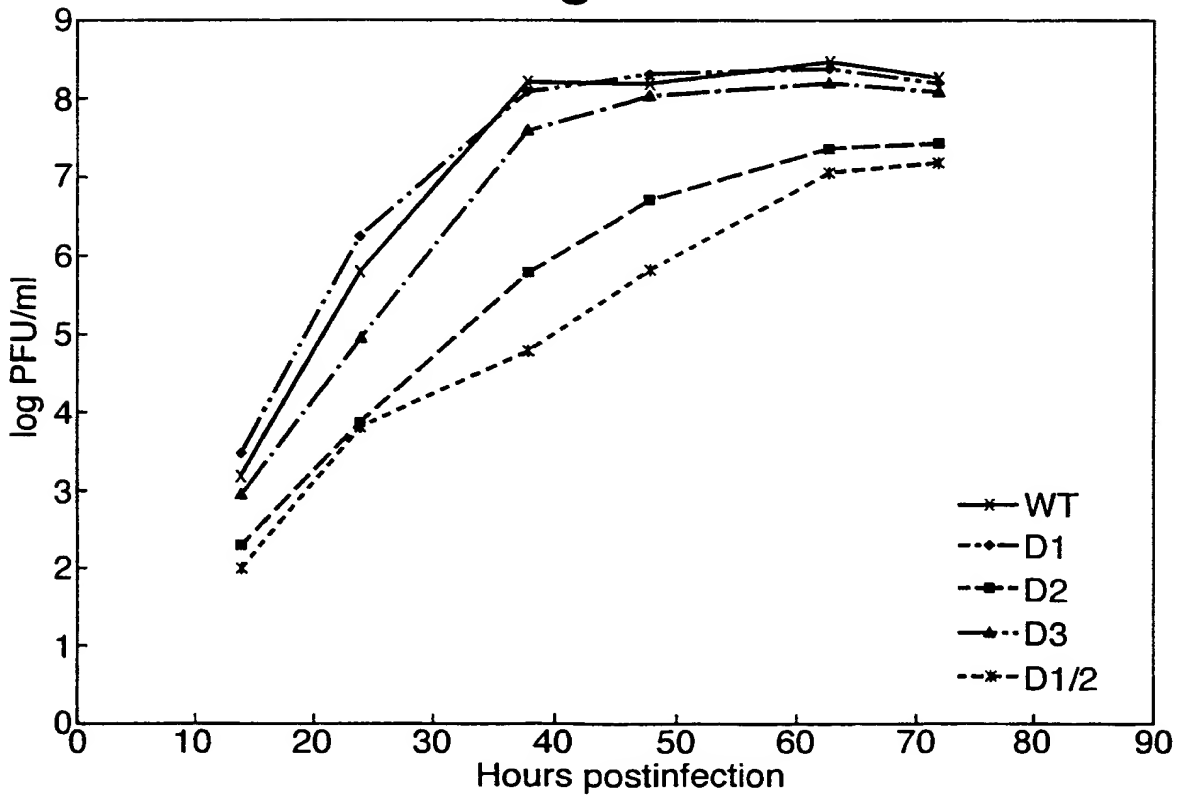

log PFU/ml
Hours postinfection
WT
D1
D2
D3
D1/2
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90

Fig.3.

```
   1  TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG
  51  GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT
 101  CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC
 151  AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC
 201  CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
 251  CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG
 301  CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT
 351  CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA
 401  GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC
 451  GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA
 501  CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA
 551  TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG
 601  CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT
 651  GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC
 701  AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG
 751  CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC
 801  TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT
 851  TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT
 901  AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG
 951  CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA
1001  TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA
1051  CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC
1101  TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
1151  GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG
1201  GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC
1251  CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT
1301  TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG
```

Fig.3 (Cont i).

1351 TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA

1401 GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC

1451 TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA

1501 ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC

1551 GGCGTCAATA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC

1601 TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG

1651 CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC

1701 AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC

1751 AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC

1801 ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT

1851 CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG

1901 TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT

1951 ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG

2001 TCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC

2051 CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC

2101 CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCT GGCTTAACTA

2151 TGCGGCATCA GAGCAGATTG TACTGAGAGT GCACCATATG CGGTGTGAAA

2201 TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG CCATTCGCCA

2251 TTCAGGCTGC GCAACTGTTG GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT

2301 ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA TTAAGTTGGG

2351 TAACGCCAGG GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGAA

2401 TTCGAAGACG CAGCAAAAGC AGGAGTTTAA ATGAATCCAA ACCAGAAAAT

2451 AATAACCATT GGGTCAATCT GTATGGTAGT CGGAATAATT AGCCTAATAT

2501 TGCAAATAGG AAATATAATC TCAATATGGA TTAGCCATTC AATTCAAACC

2551 GGAAATCAAA ACCATACTGG AATATGCAAC CAAGGCAGCA TTACCTATAA

2601 AGTTGTTGCT GGGCAGGACT CAACTTCAGT GATATTAACC GGCAATTCAT

2651 CTCTTTGTCC CATCCGTGGG TGGGCTATAC ACAGCAAAGA CAATGGCATA

Fig.3 (Cont ii).

2701 AGAATTGGTT CCAAAGGAGA CGTTTTTGTC ATAAGAGAGC CTTTTATTTC

2751 ATGTTCTCAC TTGGAATGCA GGACCTTTTT TCTGACTCAA GGCGCCTTAC

2801 TGAATGACAA GCATTCAAGG GGGACCTTTA AGGACAGAAG CCCTTATAGG

2851 GCCTTAATGA GCTGCCCTGT CGGTGAAGCT CCGTCCCCGT ACAATTCAAG

2901 GTTTGAATCG GTTGCTTGGT CAGCAAGTGC ATGTCATGAT GGAGTGGGCT

2951 GGCTAACAAT CGGAATTTCT GGTCCAGATG ATGGAGCAGT GGCTGTATTA

3001 AAATACAACC GCATAATAAC TGAAACCATA AAAAGTTGGA GGAAGAATAT

3051 ATTGAGAACA CAAGAGTCTG AATGTACCTG TGTAAATGGT TCATGTTTTA

3101 CCATAATGAC CGATGGCCCA AGTGATGGGC TGGCCTCGTA CAAAATTTTC

3151 AAGATCGAGA AGGGGAAGGT TACTAAATCA ATAGAGTTGA ATGCACCTAA

3201 TTCTCACTAC GAGGAATGTT CCTGTTACCC TGATACCGGC AAAGTGATGT

3251 GTGTGTGCAG AGACAATTGG CACGGTTCGA ACCGACCATG GGTGTCCTTC

3301 GACCAAAACC TAGATTATAA AATAGGATAC ATCTGCAGTG GGGTTTTCGG

3351 TGACAACCCG CGTCCCAAAG ATGGAACAGG CAGCTGTGGC CCAGTGTCTG

3401 CTGATGGAGC AAACGGAGTA AAGGGATTTT CATATAAGTA TGGCAATGGT

3451 GTTTGGATAG GAAGGACTAA AAGTGACAGT TCCAGACATG GGTTTGAGAT

3501 GATTTGGGAT CCTAATGGAT GGACAGAGAC TGATAGTAGG TTCTCTATGA

3551 GACAAGATGT TGTGGCAATA ACTAATCGGT CAGGGTACAG CGGAAGTTTC

3601 GTTCAACATC CTGAGCTAAC AGGGCTAGAC TGTATGAGGC CTTGCTTCTG

3651 GGTTGAATTA ATCAGGGGGC TACCTGAGGA GGACGCAATC TGGACTAGTG

3701 GGAGCATCAT TTCTTTTTGT GGTGTGAATA GTGATACTGT AGATTGGTCT

3751 TGGCCAGACG GTGCTGAGTT GCCGTTCACC ATTGACAAGT AGTTTGTTCA

3801 AAAAACTCCT TGTTTCTACT TTTAGTGAGG GTTAATAAGC TTGGCGTAAT

3851 CATGGTCATA GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA

3901 CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG

3951 AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCCAGT

4001 CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG

4051 AGAGGCGGTT TGCGTATTGG GCGC

Fig.4.

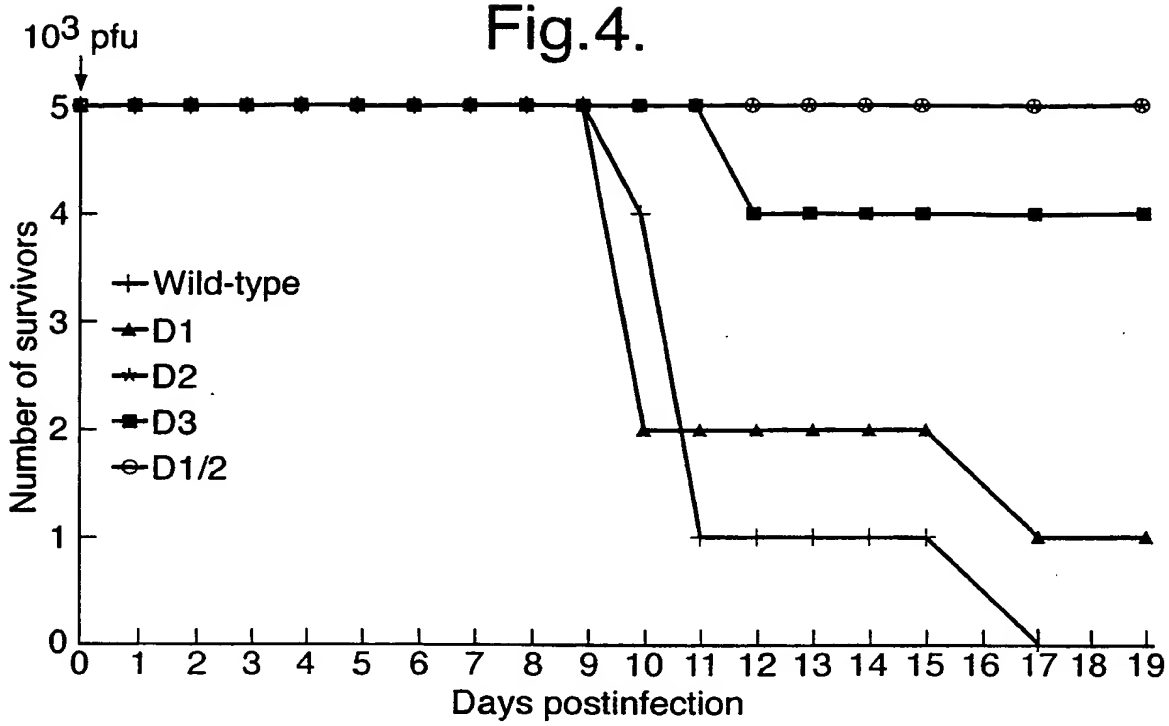

10^3 pfu
Number of survivors
Wild-type
D1
D2
D3
D1/2
0 1 2 3 4 5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days postinfection

Fig.5.

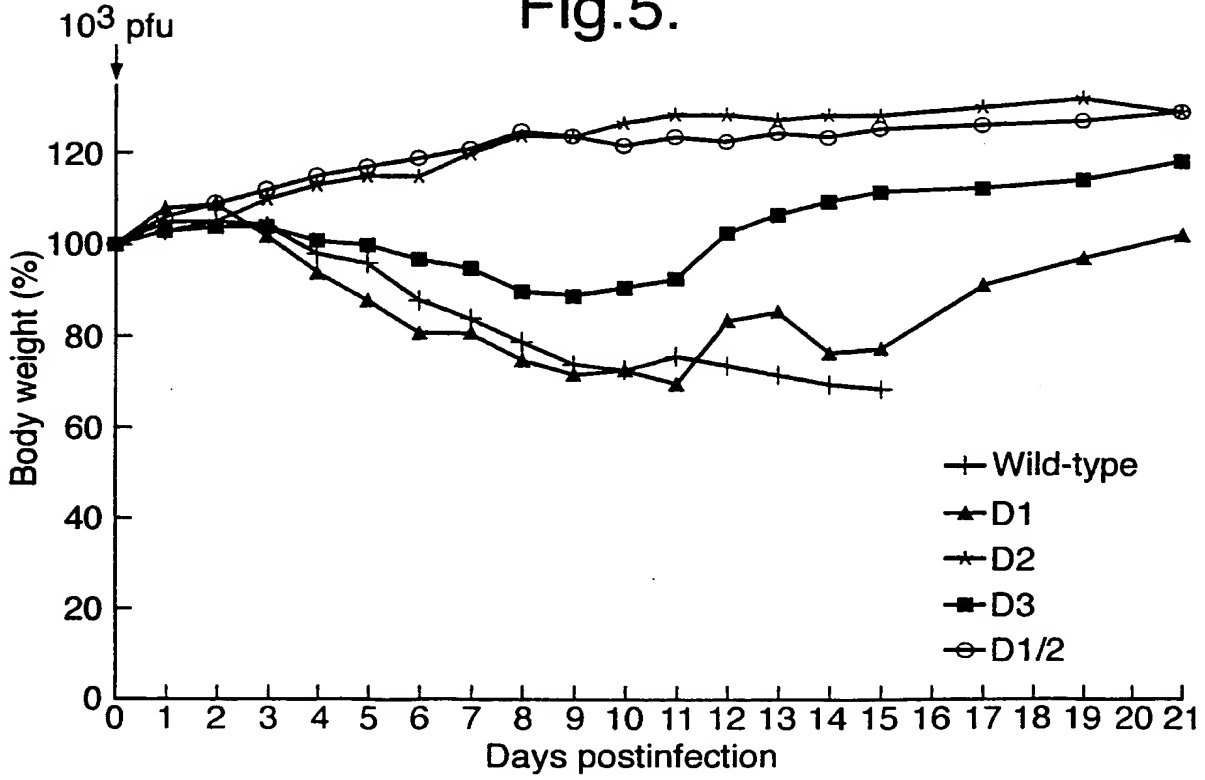

10^3 pfu
Body weight (%)
0 20 40 60 80 100 120
Wild-type
D1
D2
D3
D1/2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days postinfection

Fig.6.

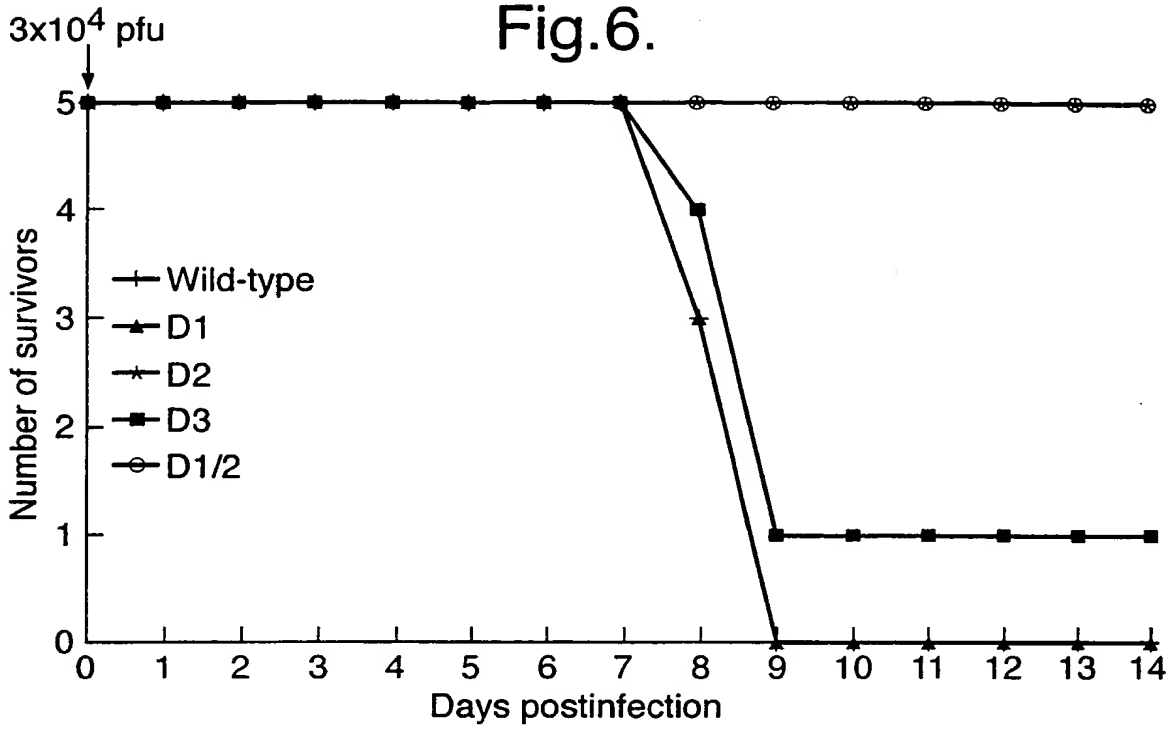

3x10^4 pfu
Number of survivors
Days postinfection
Wild-type
D1
D2
D3
D1/2

Fig.7.

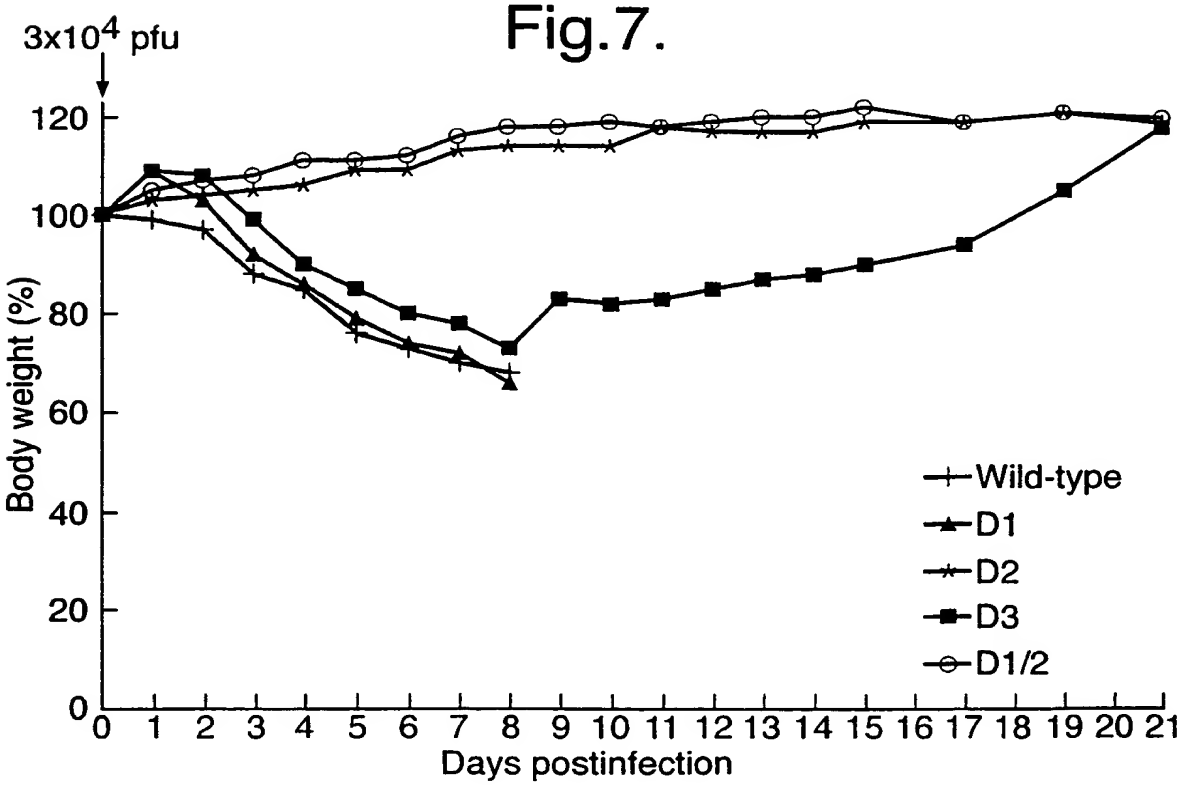

3x10^4 pfu
Body weight (%)
Days postinfection
Wild-type
D1
D2
D3
D1/2

Fig.8.

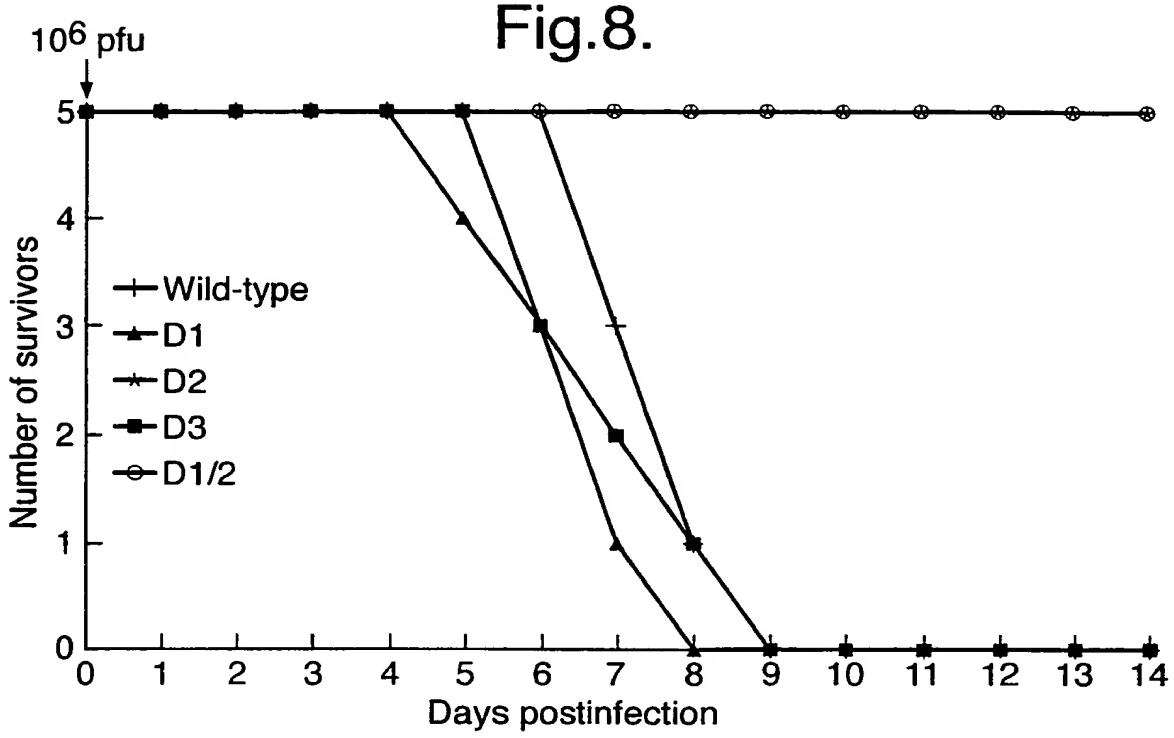

10^6 pfu
Number of survivors
Wild-type
D1
D2
D3
D1/2
Days postinfection

Fig.9.

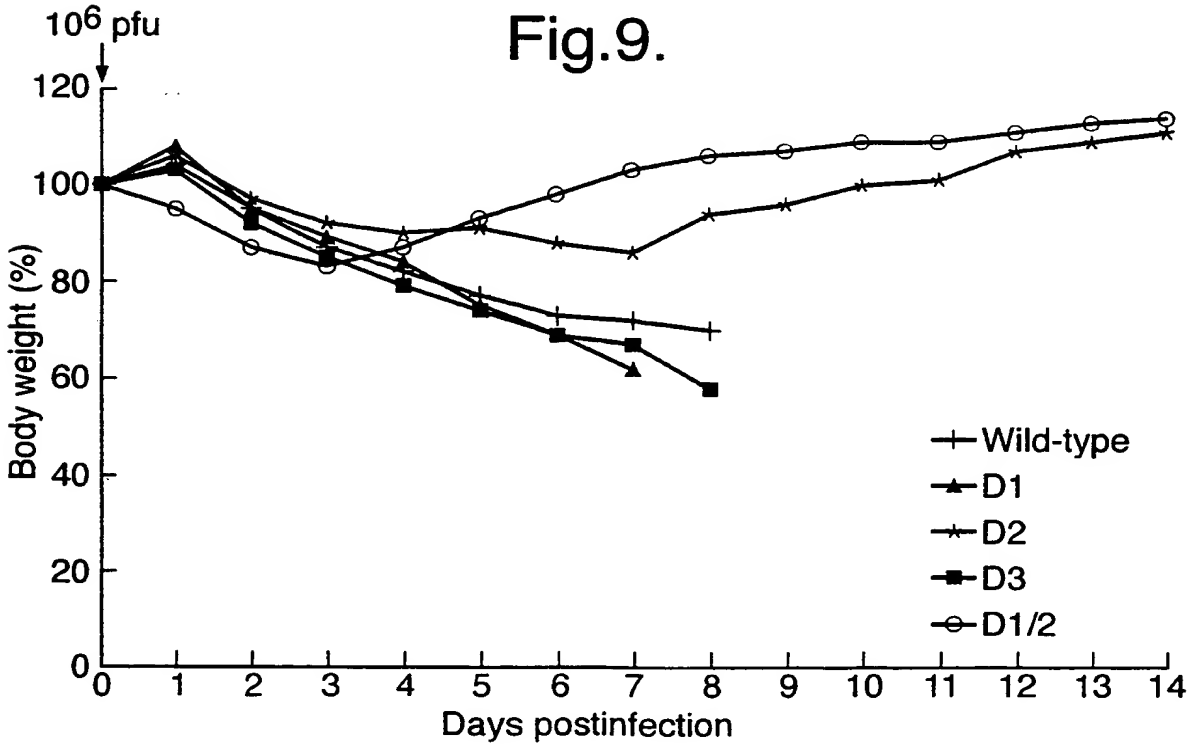

10^6 pfu
Body weight (%)
Wild-type
D1
D2
D3
D1/2
Days postinfection

Fig.10.

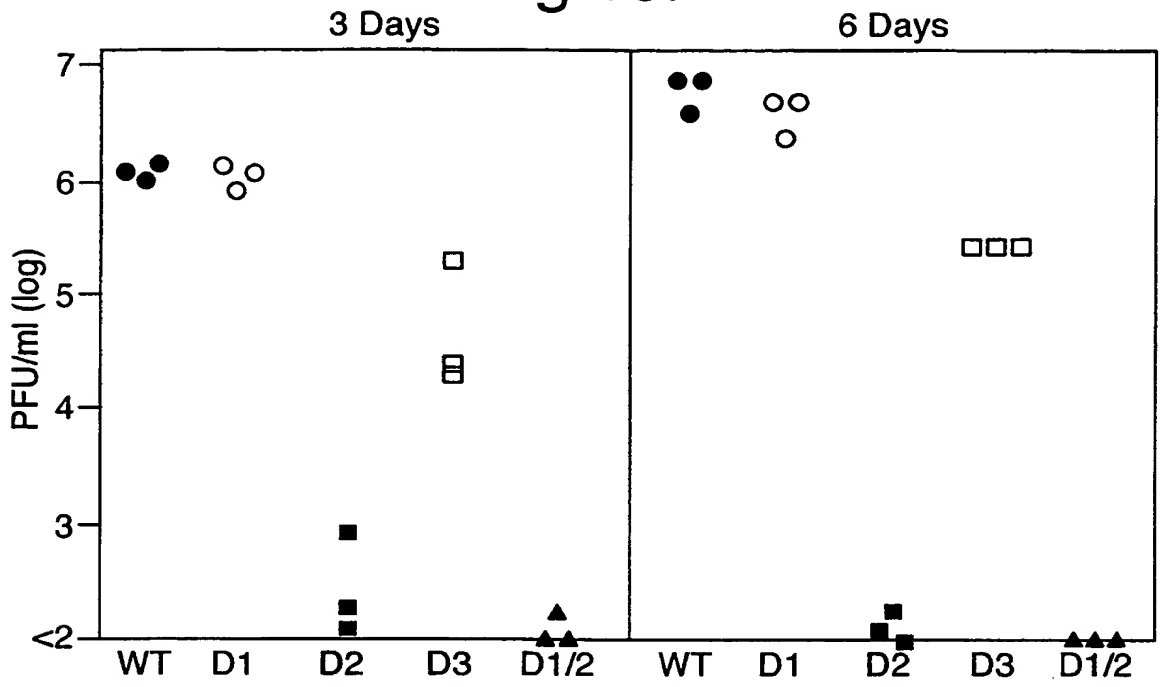

3 Days
6 Days
PFU/ml (log)
7
6
5
4
3
<2
WT
D1
D2
D3
D1/2
WT
D1
D2
D3
D1/2

Fig.11.

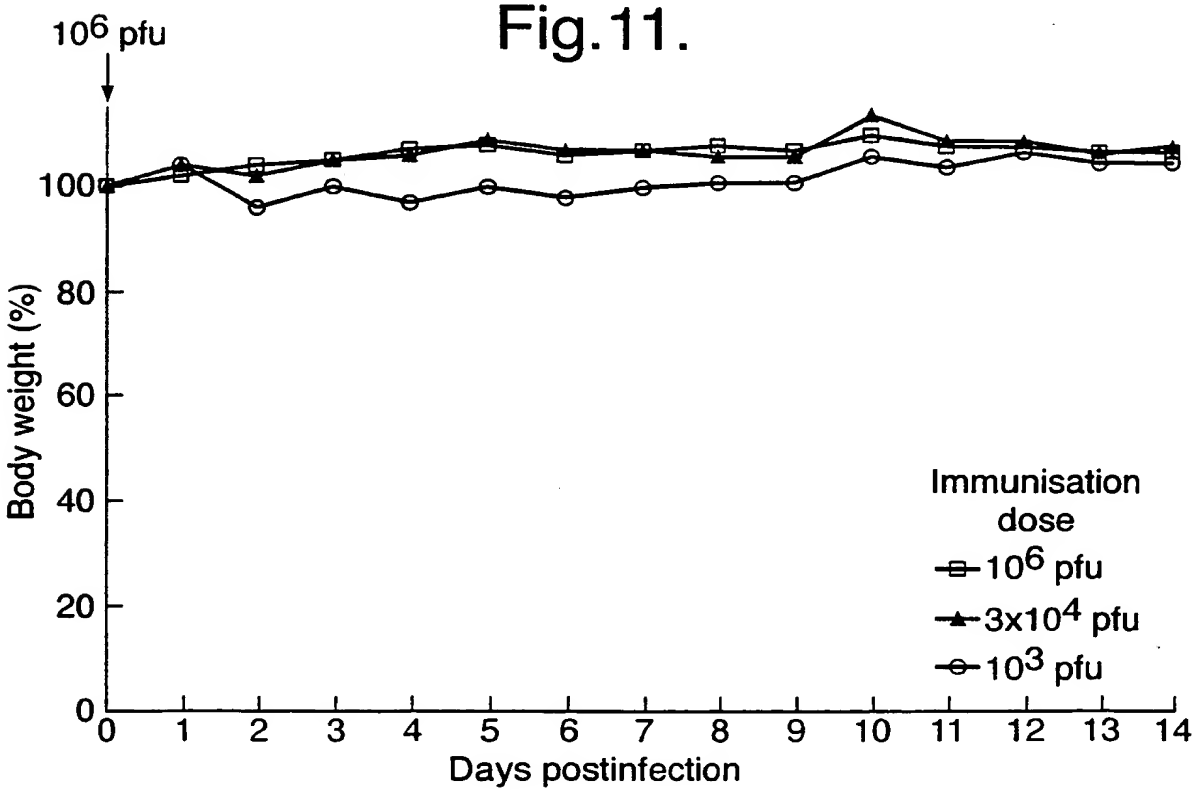
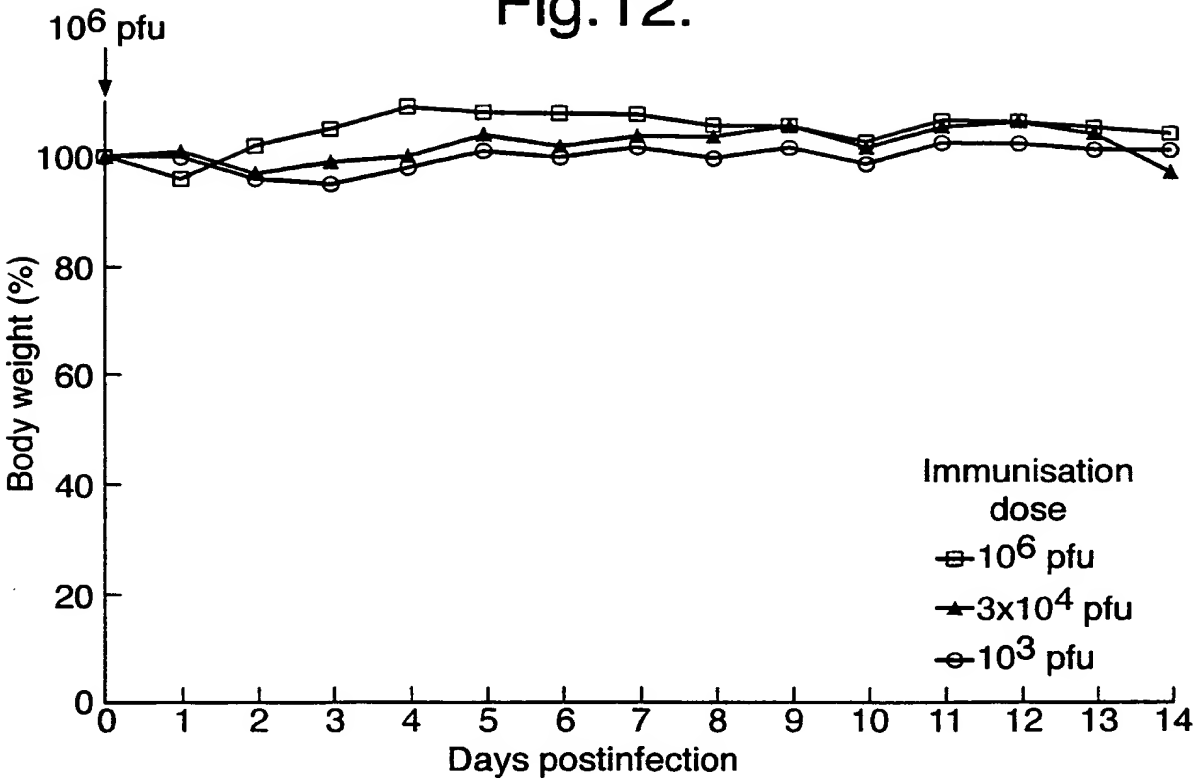

10^6 pfu
Body weight (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days postinfection
Immunisation dose
10^6 pfu
3x10^4 pfu
10^3 pfu

Fig.12.


10^6 pfu
Body weight (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days postinfection
Immunisation dose
10^6 pfu
3x10^4 pfu
10^3 pfu

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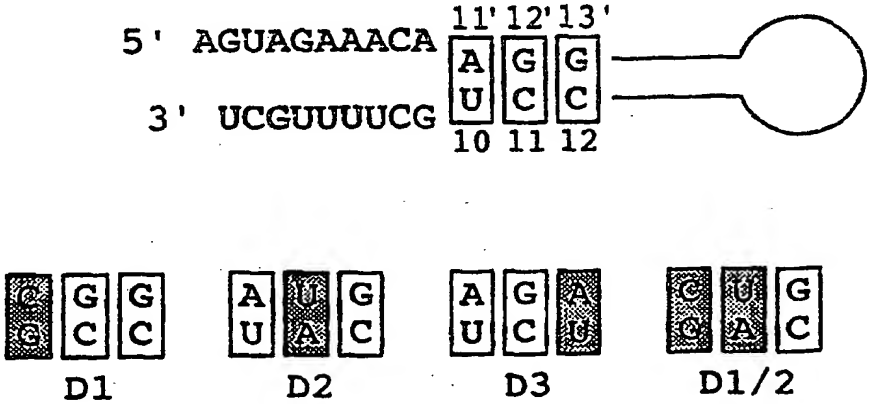
(57) Abstract

An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or a functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of said protein-coding sequence in cells infected by said virus is reduced to give an attenuated phenotype. The attenuated influenza virus can be used in a vaccine.



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CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
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CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
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(57) Abstract

An attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or a functional modification thereof, wherein said duplex region has at least one base-pair substitution such that expression of said protein-coding sequence in cells infected by said virus is reduced to give an attenuated phenotype. The attenuated influenza virus can be used in a vaccine.